The Tasmanian Devil And Its Transmissible Cancer: Physiology Of The Devil-DFTD Interaction

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…to everybody who wanders in this world,
wondering about how it works.

I. STATEMENTS AND DECLARATIONS

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The following people and institutions contributed to the publication of research undertaken as part of this thesis:

Manuel Ruiz Aravena: Contributed ideas and study design, conducted fieldwork for data and sample collection, laboratory and data analyses and wrote the manuscripts and thesis content.

Menna E. Jones: Contributed to ideas and study design, assisted with data analysis and edited the manuscripts.

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Statement of ethical conduct

The research associated with this thesis abides by the international and Australian codes on human and animal experimentation, the guidelines by the Australian Government's Office of the Gene Technology Regulator and the rulings of the Safety, Ethics and Institutional Biosafety Committees of the University.

II. PREFACE

I moved from Patagonia to Tasmania to do my PhD in the study of devils and their transmissible cancer that affects them from a "conservation medicine" approach. Soon though, after extensive and sometimes exhausting conversations and discussions with supervisors and colleagues, my project started mutating (I have since learned that is what a PhD is about). The journey took me into concepts and dimensions of the host-pathogen interactions that I had not seen before as a veterinary student or in veterinary practice: Host and pathogens are part of the same path of evolution of DNA-based life on earth, and therefore they "must" learn to live with each other.

This thesis is a written record of this adventure in Tasmania. I hope the reader will see how the approach to answer the questions mutates along the chapters and how concepts sometimes may cross disciplines. This is my little contribution to better understand our natural world, from which I have learned that even biological enemies might evolve to more benign forms.

III. ACKNOWLEDGEMENTS

The list of people that supported me in every human dimension during this scientific adventure is long. As social primates, humans depend on cooperation among individuals to optimize their fitness. In this case, the academic fitness achieved during my PhD would not have been possible without the support of many wonderful human beings.

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Research requires collaboration among organisms. As I liked to "go in between the horse's hoofs" (as we would say in Chile), the ideas that developed through these years took me to multiple fields which I could not have crossed without the help of great colleagues and collaborators:

I thank Camila, Gina, Jocelyn, Ruth, Roslyn and Bruce from the School of Medicine at UTAS and from the Menzies Institute for Medical Research. Their help and assistance were crucial to understand the aspects of devil immunology. As a field-individual, I would not have survived the demands of laboratory work without their support.

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The list continues. A huge thank you, and extreme gratitude to all the volunteers that helped me in the field. They were amazing for donating their time to assist me, no matter the weather – cold, rainy, or even as hot as an oven. I could not have conducted this research without their help.

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When you are doing field-based research, lots of things can go wrong. I was lucky to have Jenny Sprent in the team (I think every research group in the world should have "a Jenny"). Jenny, I am grateful for your work, and for keeping our research "rolling".

I thank Phil and Marita Crombie from Mistover Cottage in Yolla, and Mark and Claire Walsh from Discovery Holiday Parks-Cradle Mountain for providing accommodation and logistic support during fieldwork.

This research was conducted mostly in forestry land to which I had access thanks to Forico Ltd., Forestry Tasmania, IFarm and private landowners.

IV. ABSTRACT

The Tasmanian devil, the largest living marsupial carnivore, has been threatened with extinction because of a rare form of infectious disease, a transmissible cancer known as Devil Facial Tumour Disease. The disease emerged in the early 1990's in the northeast of Tasmania and has since spread across 90% of the wild Tasmanian devil range. Fatal in almost 100% of cases, DFTD has dramatically impacted devil numbers which have declined more than 90% at local scales and more than 80% on average. In this thesis, I apply concepts from host-pathogen theory to understand the interaction between DFTD and infected devils at the individual level. I use field-based empirical and experimental observations in two devil populations in the wild to understand the pathogenicity of the cancer and the responses from the hosts. By applying host-pathogen theory to this unconventional infectious disease, I assess patterns of tolerance and resistance in hosts which I discuss from an evolutionary perspective.

Metabolic and immune condition of devils interact with DFTD infection and progression in ways that suggest a level of tolerance and resistance to DFTD. There are consistent sex differences across metabolic and immune responses in how devils experience and respond to DFTD progression. DFTD increases the metabolic demand on infected individuals and may eventually present a constraint on the host energy budget and on the tumour itself. This is evident in an overall decline in body condition with tumour progression. Sex-differences in tolerance to DFTD are suggested by different rates in decline of body condition among the sexes, with body condition of males declining at approximately five times the rate of females at a similar cancer burden. Evidence for resistance to DFTD is provided by the positive association between the number of antigen-presenting cells in tumour tissue and the propensity of tumours to grow more slowly than expected and even to fully regress. Males and females also appear to differ in their capacity to respond to infection, with sex differences in the relationship between antibodies to DFTD and infection status.

Analysing the devil-DFTD system within the framework of host-pathogen theory helps to understand how this cancer interacts with its hosts. This study provides the first evidence of patterns of tolerance to disease progression and resistance against infection by DFTD in wild devil populations. The enhanced metabolic rates that infected devils may experience is expected to act as a constraint on both tumour and devil physiology, and

eventually favour natural selection for slower growing tumours and/or hosts with low maintenance costs. The sex differences in devils in tolerance and resistance to DFTD suggest both that the selective forces deriving from the infection affect males and females differentially, and that the selective forces acting on tumours will depend according to whether they are harboured by a male or a female devil.

The findings of this study have important implications both for the conservation of Tasmanian devils and in the broader context of disease and cancer ecology and evolution. Although Tasmanian devil populations are still declining as DFTD reaches the last naïve populations in the northwest of Tasmania, the species persistence in long-term infected areas suggests an ongoing evolutionary process between devils and DFTD. Studies to understand the genetic basis of the patterns of tolerance and resistance that are reported here are important to inform conservation management. The main conservation strategy implemented to date is the maintenance of disease-free populations in captivity and semi-wild on islands and fenced reserves to reintroduce individuals into the wild. In this context, the introduction of devils from those populations into the wild is expected affect the disease epidemic which may eventually dilute local adaptations. The ongoing evolutionary processes between devils and DFTD need to be explicitly incorporated into conservation management.

In a broader context, study of the ecological interactions between hosts and pathogens in emerging infectious diseases, such as the devil-DFTD system, provide important insights into host and pathogen co/evolution. DFTD is a young disease in evolutionary terms and provides the opportunity to observe how host and pathogen evolve on ecological time scales. By studying novel host-pathogen interactions under natural conditions in the wild we can formulate predictions about evolution of traits, test them and eventually advance theory. One of the main limitations in cancer research has been the individual nature of the disease: tumours emerge and die within the same organism. This individual-case basis of most cancers limits the study and understanding of cancer as a pathogen subject to ecological and evolutionary forces. In this context, the capacity of DFTD to be transmissible among hosts provides a unique opportunity to study cancer-host evolution *in vivo* with potential implications in the emergent field of cancer therapy such as immune and adaptive therapies.

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Chapter 1

General Introduction

CHAPTER 1. GENERAL INTRODUCTION

1.1. Ecology and evolution of host-pathogen interactions.

Biotic interactions shape life on earth by affecting the ecology and evolution of organisms (Benton, 2009). Among all interactions from organismal to population levels, host-pathogen interactions are the most ubiquitous in space and time (Dobson *et al.*, 2008). Organisms are constantly challenged by exposure and potential invasion by pathogens. Parasitism as a life form, including organisms with different levels of pathogenicity to their hosts, is the most successful and widespread life form on earth including species from all kingdoms accounting for approximately 40% of known species. Parasites[1] (and microparasitic pathogens) are defined as organisms that depend completely or in part of their life cycle on resources they extract from their host organisms. In this process, they affect the host performance and fitness in a negative way for example by decreasing fecundity or increasing mortality rates (Tompkins and Begon, 1999; Hawkins *et al.*, 2006; Robinson *et al.*, 2010; Finnerty *et al.*, 2017). With their wide distribution and effect on individual hosts, parasites have important influence on the ecology and evolution of host populations (Combes, 1996; Hudson *et al.*, 1998; Robinson *et al.*, 2010; Hollings *et al.*, 2015). The overall effect of pathogen infections on host populations depends on the ecological and evolutionary history of the host-pathogen system.

The epidemiological theory predicts infectious pathogens to become endemic in host populations over time, since high mortality of infected hosts will affect the chances of persistence of pathogens in the long term. Evolutionary new host-pathogen interactions are characterized by important declines of host populations to the point of threatening them with extinction (Altizer *et al.*, 2006; Munson *et al.*, 2008; Morley and Lewis, 2014). This epidemic pattern of infection outbreaks in naïve host populations is expected to change by natural selection operating on the host-pathogen interaction. Thus, long-term interactions can drive host-pathogen systems to full dependence and endemicity of hosts by coevolution between host and pathogens (Clayton *et al.*, 1999; Webster *et al.*, 2004; Gagneux, 2012). The wide

[1] I use the term parasite and pathogen interchangeably throughout the thesis.

distribution of host-pathogen interactions in nature suggest that coexistence is a common evolutionary outcome. Therefore, persistence of both host and pathogen in the long term will depend on the adaptive capacity to respond to natural selection imposed by and on each of their interacting populations.

1.2. Phenotypes at the front line of evolution

Natural selection acts on phenotypes, with consequences for the underlying gene frequency. Thus, phenotypes are at the forefront of natural selection as the success of genotypes in passing on their genes to the next generations depends on how well their resulting phenotypes perform in the environment the organism experience. The potential for evolution in a host-pathogen system will depend on the capacity of pathogen and host genotypes to display traits able to respond to selective pressures imposed by the interaction (Koella and Restif, 2001; Råberg *et al.*, 2009; Hayward *et al.*, 2014). Three principles are required to be met by traits to allow evolution by natural selection (Darwin, 1859): **i)** *Principle of Variation* - the trait must present variability between individuals, which means that individuals will show different values of the same trait under similar conditions; **ii)** *Principle of Differential Fitness* - the trait has to covary amongst offspring left by the individuals in the population; and **iii)** *Principle of Heredity* - the phenotype displayed by an individual and its variation is heritable and passed onto the next generation. These principles are applicable to the interacting populations of both host and pathogen, and their quantification is needed to predict the potential for evolution of the host-pathogen system.

The ecological interactions and evolutionary processes between pathogens and hosts are driven by four main traits: virulence and transmission in the pathogen, and tolerance and resistance in the host; both host and pathogen are theoretically expected to favour strategies and physiological feedbacks that may allow eventual coexistence based on the interaction of the four traits. In the pathogen, natural selection should favour optimal level of virulence to allow pathogens to colonize new hosts and replicate within without killing them before transmission to a new one (Alizon *et al.*, 2009). From the host perspective, natural selection is expected to favour genotypes which can fight the disease and clear the pathogen from the organism (resistance) or genotypes which can buffer the impacts of infection on their fitness without limiting the pathogen burden (tolerance) (Råberg *et al.*, 2009). These four traits have

adaptive value for both host and pathogen, and their ecological and evolutionary relationship will determine the epidemic patterns of the interaction in differential ways.

1.3. Tolerance to infection

Tolerance to infection is the host capacity to cope with the infection by buffering the costs of it on its fitness (Råberg *et al.*, 2009). The mechanisms of tolerance involve the capacity of the host to cope with the direct damage induced by the colonization and replication of the pathogen within the host tissues, as well as the capacity of the host to restrain its own immune response and reduce "collateral" damage to its own tissues (Medzhitov *et al.*, 2012; Soares *et al.*, 2014). An important aspect of tolerance is that it does not involve an active attack against the pathogen to restrict replication and therefore does not impose a cost on pathogen fitness (Svensson and Råberg, 2010). Tolerance may be visualized graphically by plotting the reaction norm of host health against pathogen burden (Simms, 2000; Råberg *et al.*, 2009; Louie *et al.*, 2016) (Figure 1). The slope of the resultant curve indicates the level of host tolerance, a zero slope being a completely tolerant individual and a negative infinite slope indicating a completely non-tolerant individual. Hence, the steeper the slope, the greater the impacts of pathogen burden on host fitness. Two conceptually different curves are obtained by plotting either the health level of different individual hosts at a given pathogen burden or by plotting the health level and pathogen burden of different individual hosts at different times of infection (Schneider, 2011). The first curve is called a tolerance curve, while the second provides a phase or disease curve (Jackson *et al.*, 2014). An additional host trait may be derived from this reaction norm: the intercept, which indicates the level of health or fitness of the host at a pathogen burden of zero (Råberg *et al.*, 2009). This parameter is called host vigour, and its relevance comes when individual hosts present with the same level of tolerance (slope) but have different fitness as infection progresses (Figure 1-1-B).

The application of tolerance and phase curves in studies of host-pathogen systems in wild animals has been limited, for two main reasons: i) difficulty in obtaining repeated measures of the same individual host, and ii) difficulty in standardising the pathogen burden. While the first issue may be resolved by increasing the intensity of sampling, the second is difficult to assess in the context of wild populations. Thus, it is necessary to clarify what is meant by tolerance curves measured in the context of studies of wild animal host-pathogen systems is required. I propose that tolerance curves may be built from a model that considers the pathogen burden as one of the effects, hence a "main effect" curve may be built from the model representing the reaction norm of an average individual in the population. Individuals whose health measure per pathogen burden falls above the average slope might be considered more tolerant than an average individual in the population. In contrast, phase curves are applicable to wild populations in their pure concept, being relevant for analysing individual responses to infection intensity.

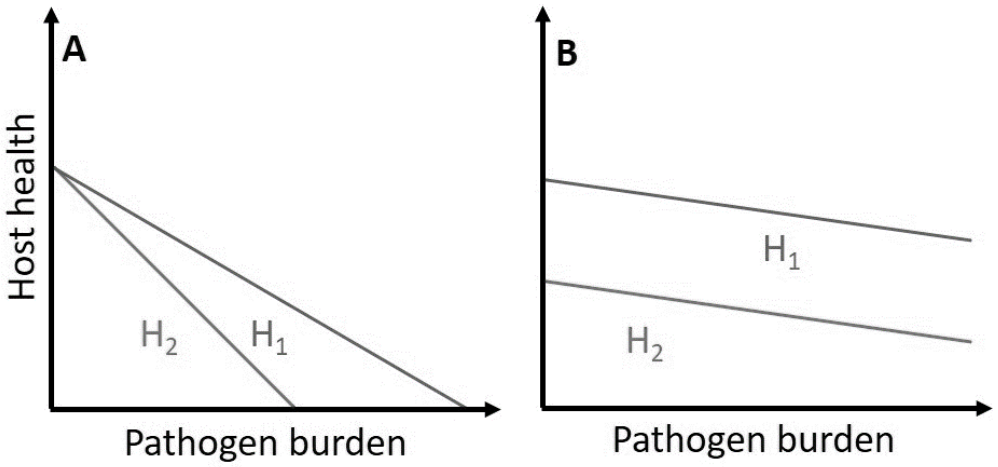

Figure 1-1 Reaction norm of host health by pathogen burden for two host genotypes. A) Both host genotypes H_1 and H_2 present same level of vigour, but differ in tolerance levels, with H_1 showing higher tolerance than H_2. B) Both host genotypes present same level of tolerance but different vigour (Adapted from Råberg et al. 2009).

1.4. Resistance to infection

Resistance refers to the mechanisms or traits that are expressed by a host once it is infected that are intended to clear the pathogen from the organism (Råberg, Graham and Read, 2009). Thus, resistance presents direct benefits to the infected host as it reduces

pathogen burden and therefore enhances host fitness. Resistance to infection include a range of energetically expensive mechanisms from generic behavioural responses, such as grooming in the case of lice-infested birds, to specific immunological responses involving antibodies that bind to specific targets on the pathogen cell surfaces, such as humoral adaptive responses to viral infections. The energetic costs of resistance derive from the maintenance and deployment of the activated immune system which trade-off with other organic functions (Lochmiller and Deerenberg, 2000; Boots, 2011; Mckean and Lazzaro, 2011; Husak *et al.*, 2016; Knutie *et al.*, 2017). For instance, the immune-suppressive effects of reproductive hormones which mediate a trade-off between defences and reproduction (Bonneaud *et al.*, 2003; Cizauskas *et al.*, 2015), may be related to the dependence of both processes on the same nutritional budget. While hosts experience costs associated with implementing mechanisms of resistance, pathogens carry direct costs to their fitness via suppression of reproductive rates. Hosts and pathogens thus both experience selective forces to maximize their fitness in infection, resulting in an arms race (Soler *et al.*, 2001; Decaestecker *et al.*, 2007).

1.5. To be or not to be… to tolerate or to resist?

Both tolerance and resistance involve costs to the host. All biological functions are sustained by a limited energetic and time budget, resulting in trade-offs between the two strategies during the organism's lifespan (Svensson and Råberg, 2010). Whether an organism will develop tolerance or resistance, or both, will be determined by the fitness costs and gains for the host genotype according to the ecological and epidemiological context (Horns and Hood, 2012; Knutie *et al.*, 2017). While both strategies may be present in a host population (Restif and Koella, 2004), and even in the same individual when tolerance and resistance share mechanisms (Mccarville and Ayres, 2018), hosts are expected to express the strategy or a combination of both which may provide it with the best cost/benefit balance (Rausher, 2001; Råberg *et al.*, 2007). It has been proposed that tolerance would be more beneficial when the risk of become infected in the host population is high as result of high disease prevalence. In this context, the cost of directly fighting the pathogen to decrease its burden (resistance) is very high because of the widespread distribution of the disease within the host population. The strategy of "learning to live with the enemy" in this context is the less expensive tactic (Boots, 2008). Although both tolerance and resistance seek to enhance the

fitness of the infected hosts, their evolutionary outcomes for the host-pathogen system differ. Resistance positively selects for countermeasures from the pathogen, perpetuating an arms race between host and pathogen. On the other hand, the evolution of tolerance may direct the host-pathogen interaction towards stable evolutionary dynamics (Roy and Kirchner, 2000; Best *et al.*, 2014). This likely outcome is based on the concept that a tolerant host increases the infectious period by living longer once infected, allowing disease prevalence to increase and thus enhancing the benefit of carrying tolerance genes. Additionally, the pathogen might decrease its virulence as a result of a longer infectious period, representing a positive feedback on host tolerance. Another benefit for tolerant host genotypes is that pathogens may be more virulent in non-tolerant hosts, and therefore reduce intraspecific competition in the host population. This dynamic would drive the whole host-pathogen system, theoretically to a commensal interaction, allowing the coexistence of both host and pathogen (Best *et al.*, 2014). The impacts of tolerance, however, are not unidirectional since an increase in survival of infected individuals may increase the chances of co-infection by multiple pathogen genotypes, which would increase competition among them, selecting for higher virulence (Susi and Laine, 2017).

1.6. The Tasmanian devil and its transmissible cancer as study case

The Tasmanian devil, a marsupial carnivore endemic to the island of Tasmania, has suffered dramatic population declines during the past 20 – 25 years. The cause is a new pathogen, a transmissible cancer called Devil Facial Tumour Disease (DFTD) (Pearse and Swift, 2006; Pye *et al.*, 2015). The first signs of devils infected with DFTD were detected in the north-east corner of Tasmania in 1996, and since then the disease has spread across the island decimating devil populations to the point that extinction was posited as a possible outcome (McCallum *et al.*, 2009). Molecular analyses revealed that DFTD originated in glial cells from a female individual devil (Murchison *et al.*, 2010).

Transmissible cancers are considered to be a rare form of infectious disease (Pearse and Swift, 2006; Metzger and Goff, 2016). Transmissible cancers differ from other infectious diseases in their aetiology, in that the infectious agent are the tumour cells themselves, which are able to be transmitted among susceptible hosts by close contact (Metzger and Goff, 2016) as allographs or transplants. In this process, for the cancer to become transmissible, at least

two conditions need to be met: i) inoculation of infective cells from an infected to a susceptible host, and ii) prevention of immune rejection from the new host. Inoculation of DFTD cells from infected to susceptible devils occurs by direct contact during agonistic interactions, including potentially devils receiving bites from tumour-laden canine teeth or devils biting into the tumours of others. Contact rates of devils increase during the mating season, when individuals bite each other more frequently, and potentially the main period of disease transmission (Pearse and Swift, 2006; Hamede *et al.*, 2008, 2013; Hamilton *et al.*, 2019). Once an individual is inoculated, immune rejection may be prevented as a consequence of multiple factors acting in conjunction: **i) Low genetic diversity of devils**. Devils present low diversity in alleles of the major histocompatibility complex, which increases the probability of tissue compatibility among individuals (Siddle *et al.*, 2007). **ii) Modulation of antigenic molecules on the tumour surface.** Tumour cells down-regulate MHC molecules on their surface (Siddle and Kaufman, 2013) preventing immune recognition by the host immune system. Under normal conditions, MHC molecules are crucial in self-recognition, thus when molecules on cell surfaces do not match those of the host, an immune response is triggered against the foreign tissue. The loss of antigens on the cell surface may be consequence of natural selection acting on the generation of "tumour escape" phenotypes (Khong and Restifo, 2002) at early stages of disease emergence. **iii) Ontogenic changes in immune condition of hosts.** Ratios between IgG and IgM, indicative of changes from generic to induced humoral responses in mammals change with sexual maturity in devils (Ujvari *et al.*, 2016). Similar patterns, have been described for cancer-related immune surveillance (Cheng *et al.*, 2017). These findings have been hypothesized as causative of detection of DFTD almost exclusively in sexually mature individuals, and not in juveniles before puberty.

At the individual level, disease progression drives its host to death in almost 100% of cases, however details about the pathogeny and pathology of disease progression are sparse. DFTD infection in hosts is characterised by the appearance of growing masses of tissue on the oral surfaces, head and neck region of Tasmanian devils. Tumour tissues usually present with ulceration through the skin or mucosal tissues which is susceptible to contamination by bacteria with the consequent infection. Once clinical signs of infection are evident, host death occurs within 6-12 months. Mortality has been proposed to be a consequence of the compromised general physiological condition of hosts by three main mechanisms as the disease progresses: 1) metastasis, 2) starvation, and 3) secondary complications (Pyecroft *et*

al., 2007). Metastasis occurs in approximately 65% of cases, compromising mainly lungs and spleen (Loh *et al.*, 2006), however, timing and likelihood of metastasis are aspects of the pathophysiology of DFTD that remain unclear. Starvation would take place when tumour location negatively affects the feeding behaviour either mechanically or chemically by releasing tumour toxins inducing anorexia. Secondary complications, such as bacterial or parasitic opportunistic infections, take place on the tumour tissue which acts as an open wound or because of immune compromise. These three conditions are not independent from each other and can function simultaneously and in synergy in the same individual host.

DFTD outbreaks follow similar trends in devil populations across its distributional range, with catastrophic effects on the abundance of the host population. The high mortality and apparent 100% of susceptibility inferred from demographic data translate to a decline in absolute devil numbers and changes in age structure 5 – 6 years after disease arrival (Lachish *et al.*, 2007). Based on these parameters, epidemiological and mathematical models suggested that extinction of infected devil populations in the wild was a possibility (McCallum *et al.*, 2009). Populations have persisted at low numbers in long infected areas, however (Lazenby *et al.*, 2018).This failure of the early models to predict the mid- and long-term demographic patterns of outbreaks could be a consequence of limited data from a single study site, lack of sophistication in the models (Wells *et al.*, 2018), or from individual heterogeneities in tolerance and/or resistance against the disease, and/or changes in virulence and transmission ability of tumours. Hamede et al. (2011) provided some of the earliest evidence of differential impacts of the disease in a population at the epidemic front in northwest Tasmania. The decline of devil numbers after disease arrival was slower than it was in populations further east. The differential epidemiological pattern in the same population has been linked to changes in the predominant tumour lineage, thus tetraploid tumours seem to be related to slower impacts on the population, while diploid tumours may be more prevalent in advanced stages of the outbreak inducing the collapse of the age structure in the population (R. Hamede *et al.*, 2015). As consequence of the clonal nature of tumours the evolution of diverse lineages may be driven by epigenetic mechanisms, while phenotypic plasticity and genetic components may play a key role in host evolution.

1.7.Signs of evolution in devils

Newly emergent pathogens represent strong selective forces for evolution by natural selection on host populations. High mortalities in naïve host populations follow epidemics by emergent pathogens and increase the natural rate of remotion of genotypes from the host populations, and therefore the selective pressures acting on them. In situations where there is a lack of phenotypic variability among hosts, the host population is expected to be driven to extinction as a consequence of its incapacity to adapt (Schloegel *et al.*, 2006). However, when heterogeneity in host susceptibility, tolerance or resistance to the new pathogens are present is present among hosts and there is a genetic basis to this variability, this process may result in an increase in the frequency of rare genotypes following the epidemic. This change in frequency of genotypes in host populations has been highlighted as key in allowing host populations to persist in the long term, by changing the "affinity" or matching between host and pathogen genotypes. In the case of the devil-DFTD system, the disease represents an extremely strong selective pressure on the devil populations because it removes up to 100% of the adult age classes which represent approximately 80% of the local populations. The strongly frequency-dependent transmission of the disease, in addition to an apparent 100% susceptibility of individual devils (Lachish *et al.*, 2007; McCallum *et al.*, 2009), hypothetically places DFTD, in the spectrum of selection strengths of pathogens on their hosts, as a case of very high selection on its devil host populations.

In the 20-25 years since the emergence of DFTD, this relatively new interaction, in evolutionary terms, between Tasmanian devils and their facial tumours has already shown signs of a rapid evolutionary response. In a first study attending the effects of DFTD in the genetic structure of devil populations, Lachish *et al.*, (2011) describe no changes in genetic diversity within populations, but differentiation among populations attributable to disease outbreak. In a genome-wide approach, disease outbreaks have been linked to changes in two regions of the devil genome (Epstein *et al.*, 2016). These regions include seven candidate genes involved in cancer and immune related functions. In a more recent study, (Hubert *et al.*, 2018) re-analysed the data set published by Epstein et al. (2016) finding 92 signatures of selection in the devil genome, including the same candidate genes identified by the previous studies, but also genes related to behavioural and cancer traits. A third study tried for the first time to link genomic information with phenotypic traits, the authors found sex differences in the capacity of the variability in SNPs to explain survival in infected individuals, with a much

greater explained deviance in females than male devils (Margres *et al.*, 2018). Two remarkable aspects of these findings are important to highlight. First is that the evolutionary response has been incredibly rapid, occurring in as little as 4-6 generations following local disease outbreak (Epstein *et al.*, 2016; Hubert *et al.*, 2018). This is much faster, for example, than evolution of European rabbits to the introduction of the *Myxoma* virus as a biocontrol agent in Australia, which is one of the classic cases of host—pathogen coevolution (Kerr *et al.*, 2015). Second, evolution has occurred on standing genetic variation, which is apparently sufficient (Epstein *et al.*, 2016; Margres *et al.*, 2018) regardless of the low genetic variability described in devils (Jones *et al.*, 2004; Morris *et al.*, 2013), which was thought to restrict their adaptive capacity (Pearse *et al.*, 2012)

1.8. Signs of evolution in tumours

The evolution of the devil facial tumours is expected to be much more restricted than evolution in the devil host as a consequence of its clonal nature. DFTD originated in mutated Schwann cells from a single female devil in the early 1990's in north-east Tasmania (Murchison *et al.*, 2010). Since its emergence, the tumour cells have replicated by clonal reproduction and passed from host to host. This clonal replication of tumours represents two major constraints for its evolution, i) intrinsic low variability among tumours (all of them have their origin in the same cell), and limited evolution potential by genomic changes. These two conditions mean that evolution in the tumour is more likely to be driven by epigenetic change than by changes in the genome (Ujvari *et al.*, 2013). Despite these intrinsic evolutionary constraints imposed by the nature of tumours, however, different lineages have been detected (Murchison et al., 2012; Pearse et al., 2012) with potential functional impacts on the epidemiological patterns of the disease (Hamede *et al.*, 2015).

Evolution of pathogens is expected to be faster than in their hosts because microparasitic pathogens have much faster intergenerational times. In the case of DFTD, however, evolution in tumours is expected to take place at a slower rate than in hosts as a consequence of their highly stable genome (Deakin *et al.*, 2012), and the epidemiological patterns of infection (Lachish et al., 2007; McCallum et al., 2007). When the disease arrives into a naïve devil population, the hosts are immediately under selection pressure, as the infection drives them to death. However, the pressure for tumours to evolve differs in its

temporal and mechanistic dynamic according to the epidemiological context (Hamede *et al.*, 2015). Thus, when susceptible hosts are abundant and disease prevalence is low, the force of selection on tumours is expected to be low. As disease prevalence increases, and therefore multiple tumours are transmitted to a naïve host by independent contacts with multiple infected hosts, selection on tumours may be driven by competition among them; later on, as host availability decreases, selection on tumours is expected to be driven by survival of infected hosts which depends on tumour virulence and host tolerance. Although, these potential mechanisms have not been explicitly tested in the devil-DFTD system, there is evidence supporting their occurrence as different tumour variants diverge as the disease continues spreading across devil populations. Murchison *et al.*, (2012) described divergences of tumour lineages based on mitochondrial DNA, with specific lineages associated to restricted geographical areas potentially emerged by selective pressures. In a second study, based on cytogenetic analyses the authors reported five different tumours strains based on their karyotype arrangements (Pearse *et al.*, 2012). Changes in the prevalence of diploid and tetraploid strains were reported in south-east Tasmania after removal of infected devils in an effort to decrease the spread of the disease in the population (Ujvari *et al.*, 2014). In a later study, Hamede *et al.* (2015) presented the first evidence of potential epidemic patterns following lineage replacement (diploid vs tetraploid) in one population of devils in north-west Tasmania. Further evidence of tumour evolution from an epigenetic perspective suggest that changes in patterns of methylation of the DNA may play a role in the evolution of tumours (Ujvari *et al.*, 2013). Finally, all the available evidence, supports the view that regardless its clonal replication, DFTD may compensate the lack of genetic-driven adaptive capacity by epigenetic plasticity which allows it to adapt and evolve in the long term.

1.9. Thesis aims and outline

As I have described above, signs of evolution in both host and pathogen are emerging as the interaction between devils and tumours progresses in time and space. The underlying mechanisms of evolution may be intrinsically different in devils and tumours; however, it has been well established that both, host and pathogen are under selective pressure. Despite this, there is limited evidence of variability in the phenotypes of the devil-tumour interaction on which natural selection may act. Immune condition of devils, and specific responses against DFTD at the individual level have been studied, but there is a lack of studies on devil traits in the context of host-pathogen ecological and evolutionary theory. In particular, no studies to date have explored differences in patterns of tolerance and resistance to DFTD at the individual and population levels.

The main aim of this thesis is to explore patterns of phenotypic interaction between the Tasmanian devil and its transmissible cancer in an ecological and evolutionary perspective. I address this aim by formulating specific questions to understand how the devil physiology relates to cancer progression (Chapters 2 – 5). I present my ecological results in an evolutionary outlook discussing them under the context of how the extended phenotype of devil-cancer interaction might drive the evolution of tolerance and/or resistance to the disease in devils. Finally (Chapter 6), I identify the caveats of this work and present some perspectives for future research from a phenotypic perspective to understand the functional biology of devils and tumours in the long-term and test predictions of selection in natural populations.

In chapter two I assess the dynamic of white blood cell counts of wild Tasmanian devils. By using a model selection approach, I assess the importance of intrinsic and extrinsic variables influencing total white blood cell counts in devils. The results show differences between male and females devils and a non-linear relationship between the white blood cell counts and disease progression.

In chapter three I explore a dimension of the cancer pathogenicity not considered in previous research: the influence of infection in field metabolic rates of wild devils. I performed field experiments using the doubly labelled water technique to assess how the energy expenditure of infected devils changed in comparison to healthy individuals in natural conditions. The results of this chapter contributed additional information about how the

cancer can impose restrictions to the energetic budget of devils and therefore compromise their performance and fitness in the wild.

In chapter four I test the general effect of cancer infection on general condition and physiology of hosts. I used the reaction norm of host body condition as proxies of health by pathogen burden to build tolerance curves at the population level. My results represent the first attempt to evaluate tolerance to cancer in Tasmanian devils and one of the very few studies assessing natural variation of tolerance to disease in natural host populations.

In chapter five I investigate signs of resistance against DFTD in devils at the population level. Specifically, I assess how the devil immune system may be influenced by or may influence the disease progression. To do so, I evaluate seasonal changes in innate and induced immune condition of healthy and diseased devils, and their relation to disease progression. In addition, I present information about immune context that may lead devils to recover from the infection and develop resistance by analysing in depth cases of naturally recovered devils. I discuss these results in the context of the evolution of tolerance and resistance against the cancer in the devil population and their potential consequences for the evolution of the system.

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Chapter 2

Dynamic of total white blood cell counts
in wild Tasmanian devils

CHAPTER 2. DYNAMIC OF TOTAL WHITE BLOOD CELL COUNTS IN WILD TASMANIAN DEVILS

2.1. Introduction

Organisms are constantly challenged by environmental conditions, and internal and external pathogens which influence their physiology and eventually their performance and fitness. The ecological pressure that pathogens represent for hosts have favoured the evolution of defensive strategies leading eventually to the development of the highly specialized immune system (Tschirren and Richner, 2006). Since pathogens are ubiquitous, the immune system is crucial for host fitness. Immune function commonly trades-off with other fitness components, such as reproduction and even counterintuitively survival (Moret and Schmid-Hempel, 2000; Husak *et al.*, 2016; Schwenke *et al.*, 2016). This compromise between immune condition of hosts and other components of fitness derives from the energetic costs associated with maintaining the cellular and molecular components of the immune system according to varying availability of resources and physiological "priorities" through the lifespan of organisms (Nelson and Demas, 1996; Nelson, 2004; Korfel *et al.*, 2015; Strandin *et al.*, 2018). As a result, the immune system can be highly affected by ontogenetic development, reproductive investment and overall energy availability among other dynamic factors, which ultimately translate to a heterogenous risk of infection within and among host populations (Altizer *et al.*, 2006).

White blood cells (WBCs) are the basic unit of the immune system, and which carry out a range of innate and adaptive immune responses. Also called leukocytes, from the greek *leuk* = white and *cyt* = cell, white blood cells lack the internal molecules that confer colour such as the case of haemoglobin in red blood cells. The total number of WBCs in a sample count is a useful generic indicator of an organism's immune competence, even though the WBCs are composed of different types, which relate to different components of the immune response (Beldomenico *et al.*, 2008a). Thus, the number of leucocytes circulating in blood is a basic and informative parameter used in veterinary medicine to assess the health status of vertebrates in generic terms (Maceda-Veiga *et al.*, 2015). Changes in WBC counts outside of

the reference intervals for a species will suggest clinical implications related to poor condition (low numbers) or reaction to infection (high numbers) (Beldomenico *et al.*, 2008b, 2009). Understanding how WBCs naturally change in wild populations in health and disease states is part of the basic knowledge to inform actions for wildlife health management (Beldomenico *et al.*, 2008a, 2009; Lazzaro and Little, 2009; Maceda-Veiga *et al.*, 2015; Peck *et al.*, 2016). Baseline knowledge is particularly important in the case of emergent infectious diseases (EIDs), because EIDs are important drivers of population declines in wildlife and are therefore of conservation concern (Daszak *et al.*, 1999; Aguirre and Tabor, 2008; Frick *et al.*, 2010; Graystock *et al.*, 2013). In this chapter, I assess how WBCs numbers in Tasmanian devils change in healthy individuals and individuals infected by a novel transmissible cancer (devil facial tumour disease; DFTD).

The Tasmanian devil, which is the largest extant marsupial carnivore on earth, has been at placed at risk of extinction as its numbers decline with the spread of DFTD (Hawkins *et al.*, 2006; McCallum *et al.*, 2007; Grueber *et al.*, 2018; Lazenby *et al.*, 2018). The transmissible cancer was detected for the first time in an individual devil in the north-east of Tasmania in 1996. DFTD was established as a transmissible cancer based on cytogenetic similarity among tumours in different hosts (Pearse and Swift, 2006). Its origin was determined as from Schwann cells of a female devil in the early 1990's (Murchison *et al.*, 2010). Transmission most likely occurs during injurious biting, which peaks during the mating season in late summer and early autumn (Hamede *et al.*, 2008). Agonistic interactions during the mating season involve male—male competition for females and intensive mate guarding of females by males during their oestrus, a process that can result in injury to both sexes (Hamede *et al.*, 2008, 2013). Biting behaviour increases the risk of infection due to contact between open wounds and tumour cells, either by inoculation of cancer cells on teeth of infected individuals or by animals biting into the tumour of another devil. Devils frequently have wounds inside their mouth and around the oral region (Hamede *et al.*, 2013). In addition to enhanced injurious biting, the reproductive season is a stressful time for both sexes, and particularly for males which lose 25% of their body weight (Jones *et al.*, 2008). High and sustained stress levels during the mating season may compromise the immune competence of devils, and act in synergy to increase the risk of establishment of tumour cells transferred to a new host. Studies exploring the natural variation of immunity in wild devils are sparse, reference intervals for the species have recently been published (Peck *et al.*, 2015).

We first explore the influence of ecological variables on the total white blood cell counts of wild Tasmanian devils. Using two modelling approaches based on linear mixed models and generalized additive mixed models, we assess the nature of the relationship between intrinsic and extrinsic variables and the WBC of devils. Second, we present the relationship between the progression of infection with DFTD and total WBC counts and hypothesise how the immune competence of individual hosts may be both the cause and the consequence of health status of Tasmanian devils.

2.2.Methods

2.2.1. Field data and sample collection

Two study sites of 25 km^2 each were established in northwest Tasmania, Australia, in the localities of West Takone (379369 E / 6440624 S) and Wilmot (426417 E / 5414182 S). The devil populations at the two sites were sampled at three-month intervals, between February 2015 and 2017 (February 2016 was not conducted), to address seasonal and physiological changes associated with reproduction. The resulting data set comprised 12 trapping sessions for the Takone site, and 8 sessions for Wilmot. Each field session consisted of 10 continuous days of trapping in which 40 custom-built carnivore traps were deployed. Upon first capture, each individual devil was permanently marked with a subcutaneous microchip, and sex and age (using tooth wear, accurate to 3 of 5 years lifespan) were determined. Body mass and head width (maximum width across the zygomatic arch), as a linear measurement of skeletal size, were recorded. Head width was also used as a continuous indicator of temporal and physiological age. For individuals presenting with clinical signs of infection with DFTD, a photo-identification of individual tumours was captured, and the maximum length, width and depth of tumours measured, volume being calculated later using the formula for ellipsoid volume. Infected devils often presented with more than one tumour in different locations on the body at the same time. In these cases, total tumour volume was calculated by summing the volumes of all tumours present in the host at the time of observation. A body condition index was calculated from the allometric relationship between body mass and head width (Ruiz-Aravena *et al.*, 2018).

Blood samples to perform white cell counts were collected from ear veins. 200 ul of blood were collected in EDTA by puncture of the ear vein with a hypodermic needle. White

blood cell counts were performed in a Neubauer chamber within 10 hours from sample collection. 50 ul of blood were diluted in 950ul of crystal violet (0.2%). The total number of cells were counted in five large squares of the Neubauer chamber, and then divided by 5 to perform calculation of number of cells per ml of whole blood.

2.2.2. Statistical analyses

We evaluated the relative influence of external and intrinsic variables on the total counts of WBCs in Tasmanian devils by using linear mixed effects models (LMM) and generalized additive mixed models (GAMM) to account for non-linear relationships among variables. In both analyses, we applied a model selection approach to discard variables with limited explanatory capacity. In the case of LMM, the model selection was performed based on the AIC values obtained for different model formulations. In the case of the GAMM, we formulated the same models used in the LMM framework and conducted a slightly different approach for model selection based on AIC used for the LMM. The GAMM formulation allows for penalization of the smooth terms included in the model which allows shrinking of the smooth term to zero if the term does not contribute to the model. In this way, if a variable has effective degrees of freedom approximating zero, the model is refitted without that smooth term. On the other hand, values of effective degrees of freedom also help assess the nature of the relationship among variables. Values close to 1 support a linear relationship between variables while values above 1 suggest a strong non-linear relationship.

The response variable (white blood cell counts) was log-transformed to meet normality assumptions. Therefore, I used a Gaussian error distribution in both, the LMM and GAMM formulations was used. Predictor variables in both LMM and GAMM included: individual sex, head width as a proxy of body size and age, total tumour volume in mm^3, ratio between total tumour weight and body weight (T/B), body condition, and day of the year as a proxy for seasonality. Day of the year was modelled as angles to account for circularity of the variable. The two-way interaction terms between sex and the rest of the variables were also included as predictors, to account for intrinsic physiological differences between sexes that may influence the WBC numbers (e.g. endocrine profiles) (Pennell *et al.*, 2012; Roved *et al.*, 2017). Tumour burden was included in the models as either total tumour volume or T/B avoiding including both due to their correlation. Finally, individual devil ID was included as a random effect to account for non-independence of observations derived from multiple

measures from the same individual. All statistical analyses were conducted in the R environment, using the libraries “nlme” and “mgcv” for the LMM and GAMM, respectively.

2.3. Results

A total of 247 individual values for white blood cell counts from 151 individual Tasmanian devils were included in the analyses. The data included observations collected between November 2015 and May 2017. Overall, 41 observations (16.59%) fell outside the reference intervals described for wild Tasmanian devils by Peck *et al.* (2015). Of those 41 observations, 56.09% were recorded in individuals without clinical signs of infection by DFTD. No sex bias was evidenced among the 35 individuals presenting leucocytosis (WBC above the reference interval) ($\chi^2 = 0.14$, $df = 1$, $p = 0.7$). In contrast, all the individuals with leukopenia (n = 6) were females (Figure 2-1 and 2-2).

The best LMM formulation (delta AIC <2) explaining white blood cell counts included the variables head width and the ratio between tumour and body weight (Table 2-1) and explained 24.7% of the null deviance. The total number of white blood cells declined with increasing head width (Figure 2-1A). T/B showed an opposite relationship, with white blood cell counts increasing with T/B (Figure 2-1B).

The GAMM formulations performed better than the LMM in their explanatory capacity of the data. The GAMM with most support presented an AIC 9.67 unit lower than the most supported LMM formulation and accounted for 42.7% of the null deviance, contrasting the 24.7% from the best LMM. This better performance of the GAMM over the LMM formulations suggests strong non-linearities in the relationship between the variables. The selection of smooth terms by shrinkage identified head width, T/B, and the interactions of sex and day of the year and sex with body condition as the most important explanatory variables (Table 2-2). Head width presented an almost linear negative relationship with white blood cells (Figure 2-2A). In contrast, T/B presented a non-linear saturating curve relationship (Figure 2-2B) with WBC increasing as the tumour weight relative to body weight increased. The relationship of body condition and day of the year with cell counts varied with sex. Body condition was associated with WBC only in females and was linear, in contrast to males in which there was no relationship (Figure 2-2C). Day of the year presented a slightly declining non-linear relationship to WBC in females, while in males there was no relationship of day of year with WBC (Figure 2-2D).

Table 2-1 Results of the model selection on Linear Mixed Model formulation for total white blood cells in wild Tasmanian devils.

Predictor	Full Model		Best model	
	Estimate	**SE**	**Estimate**	**SE**
Intercept	9.575	1.205	8.969	0.688
HW	-1.220	0.602	-0.908	0.341
Tvol	0.008	0.008	-	-
T/B	14.853	3.700	17.939	2.133
SexM	-0.143	1.713	-	-
BC	-0.322	0.135	-	-
Day	-0.039	0.016	-	-
HW:SexM	0.088	0.851	-	-
BC:SexM	0.331	0.189	-	-
Tvol:SexM	-0.001	0.013	-	-
T/B:SexM	1.731	5.638	-	-
Day:SexM	0.026	0.025	-	-
AIC	-223.28		-258.07	
Deviance	19.7		24.7	

Estimated coefficients from the full and most supported model formulations are shown under the column "terms". AIC is the Akaike information criterion, and D^2 is the percentage of explained deviance. WBC = white blood cell count, HW = head width, Tvol = total tumour volume in mm^3 (log-transformed), T/B = ratio between total tumour weight and body weight, SexM = sex (male), BC= body condition, Day = day of the year.

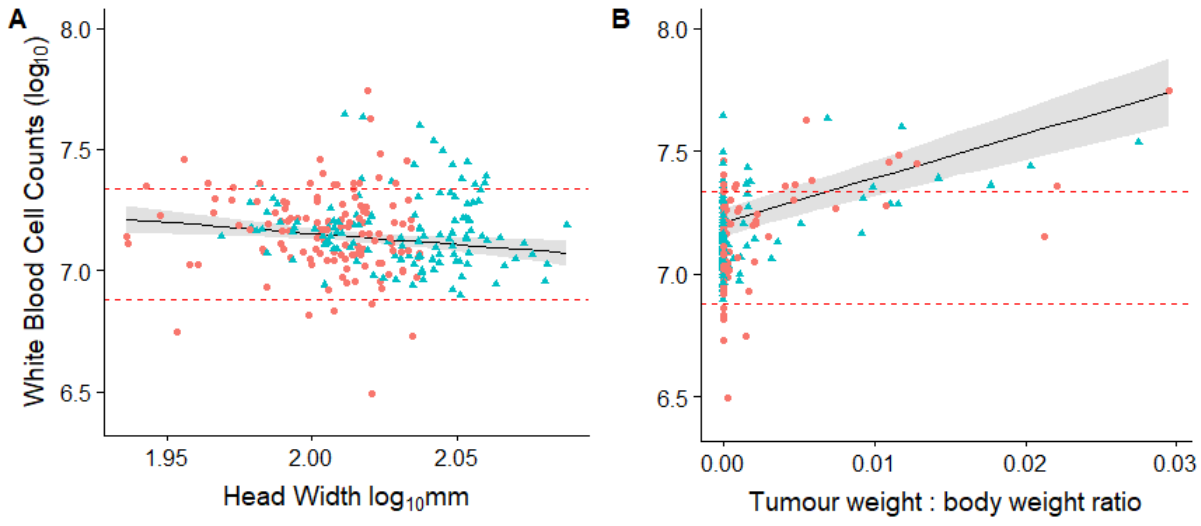

Figure 2-1 Relationship between white blood cell counts and head width (A) and white blood cell counts and the ratio between total tumour weight and body weight (B) estimated from the most supported LMM formulation. Points in plots represent observations for males (green triangles), and females (red circles). Red dashed lines represent the reference intervals described by Peck et.al. (2015) for total white blood cell counts in wild Tasmanian devils.

Table 2-2 Results of the model selection on Generalized Additive Mixed Model formulation for total white blood cells in wild Tasmanian devils.

Predictor	Full Model			Best Model		
	Estimate	**SE**	***edf***	**Estimate**	**SE**	***edf***
Intercept	7.142	0.013	-	7.146	0.013	-
s(HW)	-	-	1.371	-	-	1.266
s(Tvol)	-	-	≈ 0	-	-	-
s(T/B)	-	-	2.575	-	-	2.451
SexM	0.039	0.020	-	0.032	0.020	-
s(BC)	-	-	≈ 0	-	-	-
s(Day)	-	-	≈ 0	-	-	-
s(HW) : SexM	-	-	≈ 0	-	-	-
s(HW) : SexF	-	-	≈ 0	-	-	-
s(BC) : SexM	-	-	≈ 0	-	-	≈ 0
s(BC) : SexF	-	-	0.790	-	-	0.864
s(Tvol) : SexM	-	-	≈ 0	-	-	-
s(Tvol) : SexF	-	-	≈ 0	-	-	-
s(T/B) : SexM	-	-	≈ 0	-	-	-
s(T/B) : SexF	-	-	≈ 0	-	-	-
s(Day) : SexM	-	-	≈ 0	-	-	≈ 0
s(Day) : SexF	-	-	1.673	-	-	1.673
AIC	-265.26			-267.49		
Deviance	41.0			42.7		

Estimated smooth terms from the full and most supported model formulations are shown under the column "terms". S_i represents the cubic regression spline for the respective variable, df are the effective degrees of freedom, AIC is the Akaike information criterion, and D^2 is the percentage of explained deviance. WBC = white blood cell count, HW = head width, Tvol = total tumour volume in mm^3 (log-transformed), T/B = ratio between total tumour weight and body weight, SexM = sex (male), SexF = sex (female), BC= body condition, Day = day of the year.

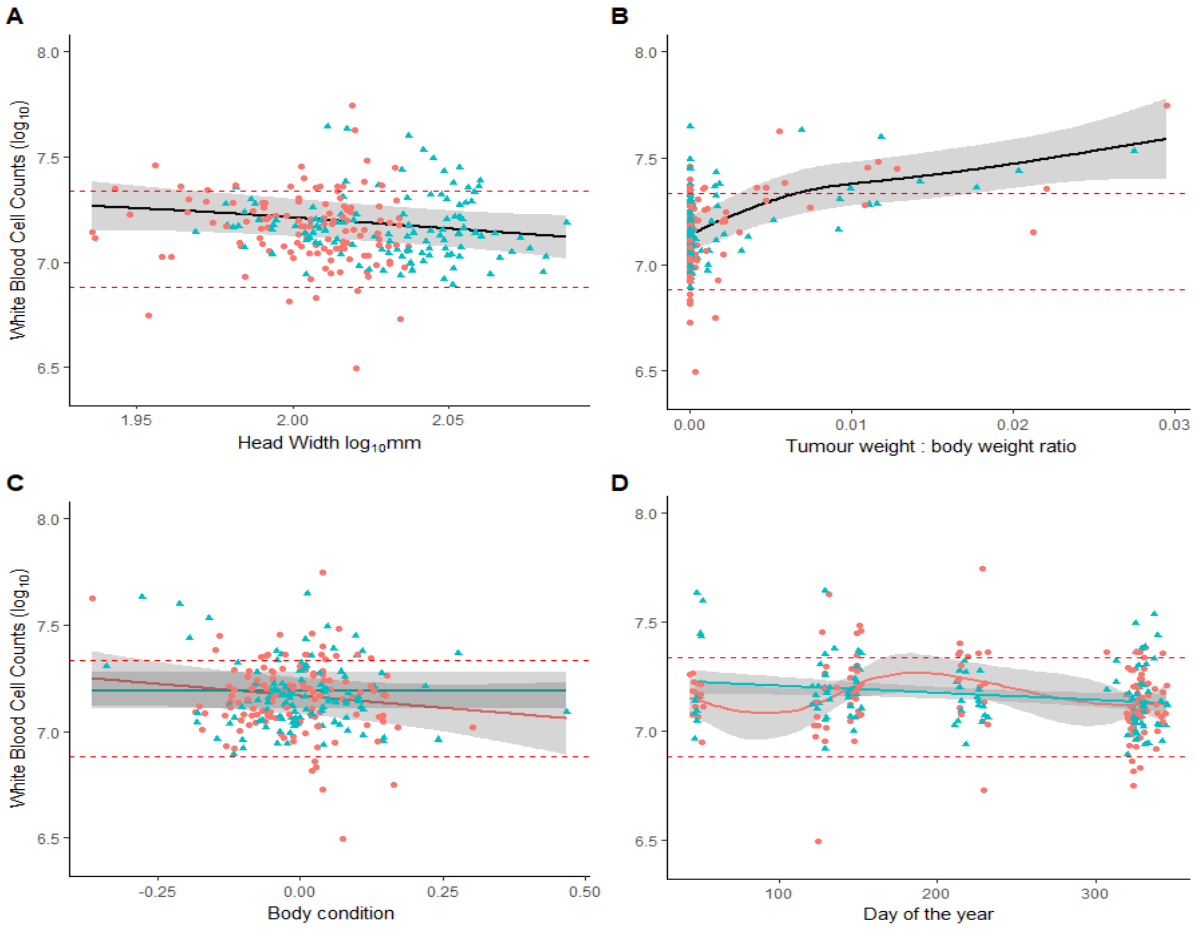

Figure 2-2 Relationship between white blood cell counts and predictor variables estimated by the most supported GAMM formulation. A) head width, B) ratio between total tumour weight and body weight, C) Body condition by sex and, D) Day of the year by sex. Points in plots represent observations for males (green triangles), and females (red circles). Red dashed lines represent the reference intervals described by Peck et.al. 2015 for total white blood cell counts in wild Tasmanian devils.

2.4. Discussion

We present an evaluation of the importance of intrinsic and extrinsic factors influencing the total number of white blood cells in wild Tasmanian devils. The methodological approach applied shows that white blood cell numbers in wild Tasmanian devils are influenced by internal devil attributes (e.g. sex), environmental conditions (e.g. seasonality) and by the interaction of individual devils and the recently emerged transmissible cancer, the devil facial tumour disease (DFTD). The results show that white blood cell counts of wild devils decrease with age in both males and females but follows different seasonal patterns in each sex. In females, WBCs increase to a peak in early winter and then decrease through spring and summer into autumn. Males do not experience changes in white blood cell counts through the year. Differences between the sexes were detected also in the relationship between body condition and cell counts. There was almost no relationship in males, while in females the number of cells decreases as body condition increases. Finally, there is a strong non-linear relationship between white blood cell counts and the ratio of tumour and body weight, with and overall increase in cell counts as T/B increases. These findings support the idea that the immune status of wild Tasmanian devils is dynamic and influenced by environmental and physiological conditions.

Total white blood cell counts in devils show a general decline with age (head width) in consistency with previous studies in captive (Hope and Peck, 2016; Stannard *et al.*, 2016) and wild Tasmanian devils. This pattern may not have clinical implications for health as the total range in white cell numbers between the smallest and largest devils (head width) was within the reference intervals for the species (Peck *et al.*, 2015), however it highlights the need for stablishing differential reference values for different age classes. The pattern One potential explanation for the decline of WBC as skeletal size of individuals increases might be trade-offs associated with ontogenetic stage in energetic investment in immune function, growth and reproduction or effects of senescence (Norris and Evans, 2000; Valtonen *et al.*, 2010; Schwenke *et al.*, 2016).

White cell counts increase with pathogen load (tumour to body weight ratio) in both model types (LMM, GAMM), but the GAMM formulation showed non-linear detail in the pattern of response with increasing pathogen load. There was a sharper increase in the number of white blood cells in the early stages of infection, when tumour weight represents

approximately 1% of the body weight of the infected devil in consistency with previous studies (Peck *et al.*, 2016). During this early phase of infection, WBCc reach levels above the upper limit of the reference intervals for the species, indicating leucocytosis. This increase in the number of WBC is expected to be related to the progression of DFTD as previous studies did not find differences between healthy individuals and devils with wounds, however DFTD infected devils presented significantly higher number of neutrophils. The higher WBC counts as tumour burden increases, may be driven initially by the damage of host tissues from mechanical invasion by DFTD tissues, and at late stages, by secondary bacterial colonization of ulcerated tumours (Loh *et al.*, 2006; Peck *et al.*, 2016).

GAMM formulations performed better in capturing the relationship between WBC and body condition of devils and seasonality. Day of the year and body condition, and their interactions with sex, were part of the final GAMM structure. For body condition, the GAMM did not show a relationship with WBC in males, while in females it did. Two potential explanations may be drawn from the negative relationship between WBC and body condition of females, although this response is not expected to have clinical relevance for individual health as the values fall within the reference intervals described for the species. First, if body condition is mainly driven by exposure to pathogens, individuals with high body condition may be less exposed to infections, and therefore WBC numbers maintain low (Debeffe *et al.*, 2016). Another potential explanation rises if body condition positively correlates with other fitness components that trade-off with immunity (Husak *et al.*, 2016). For instance, individuals in better body condition may invest more in reproduction, which has been largely documented to have immune-suppressor effects, and therefore present lower WBC counts (Trigunaite *et al.*, 2015; Schwenke *et al.*, 2016). Sex differences were also detected in how WBC numbers vary along the year. WBC in females varied significantly throughout the year with the lowest level in early autumn, then highest in early winter. This pattern in females is likely to reflect reproductive investment and may be mainly driven by changes in the number of circulating neutrophils as previously reported (Peck *et al.*, 2015). Mating season in devils takes place in late summer and early autumn, females experience low costs during pregnancy and early stages of young in the pouch, which substantially increase as denned young grow faster towards independence in early summer. In contrast to females, reproductive patterns in male devils do not seem to be seasonally driven (Keeley *et al.*, 2012), and therefore they may not experience important variation in the costs of maintenance along the year, which explain the lack of seasonality in their WBC numbers, however this findings

contrast with previous studies that report seasonal changes in WBC in adult males. One potential explanation for this contrasting findings is the methodological differences in the treatment of season as factor in the study by Peck *et al.*, (2015) or as a continuous variable in this study.

Maintenance and deployment of a competent immune system is an expensive condition for organisms, but also critical for survival (Lochmiller and Deerenberg, 2000; Bonneaud *et al.*, 2003; Beldomenico *et al.*, 2008b). Both phenotypic and genetic costs that influence physiological trade-offs have been proposed and demonstrated to be present when immune activation takes place (Promislow and Harvey, 1990; Martin *et al.*, 2003, 2008). These costs are related, for instance, with increased metabolic activity by immune cells, altered priorities for energy allocation, and damage in host tissues if there is exacerbated or uncontrolled immune activation. The magnitude of seasonal variation experienced by female devils represents a change of approximately 8x10^7 cells x ml^{-1} which equals to 40 – 50% the normal number of white blood cells in a healthy individual. Thus, in a scenario where defences are energetically and ecologically expensive, and investment on them depends upon other expenses such as reproduction, it is possible to suggest that seasonality in female devils might have a cost equivalent to 50% of their energetic budget for defences, however this costs may be different if the changes in number of cells are compensated by other components of the immune system (Jackson *et al.*, 2011; Gilot-Fromont *et al.*, 2012). Further studies including indicators of the innate and adaptive branches of the immune system are required in order to understand overall changes in the immune condition of Tasmanian devils in the wild.

The sex differences found in the relationships between WBC and both body condition and seasonality indicate that male and female devils may experience the environment differently influencing their physiology. Because of this, the costs of immunity and therefore the defensive strategies such as tolerance and resistance to infections are expected to vary between males and females. Evidence of differences between male and female devils is consistent across multiple analytical approaches. Sex bias in the levels of tolerance to DFTD have been recently reported, with females presenting patterns of higher tolerance than males. At the genomic level, female survival and potentially resistance to cancer has been linked to variability in the genome (Margres *et al.*, 2018). Finally, sex differences suggesting that the antigenicity of DFTD may be different in males and females confirm that the immune

response against the cancer varies between the sexes (Chapter 5). This combined evidence suggests that the host-pathogen interactions should vary in outcome according to whether a pathogen is harboured by a male or a female devil. This, in turn, is expected to differentially drive the evolutionary interactions between host and pathogen and to potentially be a source of divergent sex-biased defensive strategies (Roved *et al.*, 2017).

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Chapter 3

The metabolic cost of cancer
in the endangered Tasmanian devil

CHAPTER 3. THE METABOLIC COST OF CANCER IN THE ENDANGERED TASMANIAN DEVIL

3.1. Introduction

Emerging infectious diseases are recognised as a major threat to biodiversity (Daszak et al., 2000; Cunningham et al. 2017). Although viruses and bacteria are thought of as the main pathogens of concern in wildlife conservation (Daszak *et al*, 2000; Smith *et al*, 2009; Frick *et al* , 2010; Kilpatrick *et al*, 2010; Robinson *et al*, 2010), other non-traditional diseases, such as cancer, are increasingly accepted as relevant in the conservation of natural populations (McAloose and Newton, 2009; Metzger and Goff, 2016). Even though cancer is widespread across metazoan taxa and has been linked to dramatic declines in species abundance (Martineau *et al.*, 2002; Lachish *et al.*, 2007), it has been neglected for a long time in conservation sciences because it has been thought as an individual condition until recent discoveries of transmissible cancers in nature (Metzger *et al.*, 2015; Ruth J. Pye *et al.*, 2015; Metzger and Goff, 2016; Madsen *et al.*, 2017).

The Tasmanian devil facial tumour disease (DFTD) is a transmissible cancer that has dramatically reduced the abundance of Tasmanian devils (Lazenby *et al.*, 2018). This cancer increases the mortality of reproductively active individuals as a consequence of metastasis to vital organs, interference with feeding behaviour or secondary infections (Loh *et al.*, 2006). At the host level, cancer cells can be thought of as parasites, since their survival and fitness depend on their capacity to extract and assimilate resources from the host (Ujvari *et al.*, 2016). The parasite replication (i.e. tumour cell proliferation) creates a permanent demand for resources, which are taken up from the host's budget (den Broeder *et al*, 2001; DeBerardinis *et al*, 2008; Vander Heiden *et al*, 2009; Luo *et al*, 2014; Hicks *et al*, 2018). As cancer cells develop mechanisms for immune evasion (Siddle *et al.*, 2007, 2013; Pardoll, 2015), they are often not recognised by the host immune system, and therefore are metabolically treated as a host tissue that is undergoing growth. As host energy inputs are limited, it follows then that individuals with greater energy resources allocated to growth would have less energy available for other functions such as reproduction, performance or

even maintenance costs. In this scenario, hosts may have at least two strategies in order to maintain a positive energy balance: (i) re-allocate energy among competing functions, and/or (ii) to adjust metabolic ceilings (limits) to increase energy inputs (Piersma and van Gils, 2011). The energetic dimension of diseases in wildlife is thus a key aspect of the pathogenic cascade of physiological effects at the individual level (Martin *et al.*, 2018).

Here we study the field metabolic demand of Tasmanian devils infected with DFTD (Loh *et al*, 2006; R. J. Pye *et al*, 2015). We predict that infected individuals should present with higher Field Metabolic Rates (FMR) than healthy individuals as a consequence of the energetic costs of the growing tumour.

3.2. Methods

3.2.1. Field metabolic rate

We measured FMR by the Doubly Labelled Water method (DLW, Nagy et al. 1983). Eighteen wild devils (females = 9, males = 9) were trapped during autumn 2016 in an infected population in north-west Tasmania (379698 E / 5439909 S), 8 of which had DFTD (females = 5, males = 3). Although the 18 devils were injected with DLW following capture, only 7 were recaptured during the study period of ten consecutive days.

At the first capture, each devil was injected subcutaneously with a solution of DLW, after which it was left in the trap for at least 4 hours to allow for isotope equilibration. Blood samples were collected before injection, 4 to 6 hours after injection and a final sample when the animal was recaptured within a 10-day window. We used the two-sample approach to estimate the isotope turnover, evaluating its background, initial (equilibration), and final levels in blood. Whole blood was centrifuged, and the serum extracted and frozen in vacuum-sealed tubes for posterior distillation. All individuals were released at the place of capture after injection of DLW and after obtaining the blood samples.

3.2.2. Isotope sample analysis

Serum samples were vacuum distilled according to Nagy (1983). The isotopic composition for $d^{2}H$ and $d^{18}O$ in the distilled samples was determined on a MultiFlow headspace analyser coupled to an Isoprime 100 continuous flow isotopic ratio mass

spectrometer. To do so, the total of the sample was transferred from the capillary into a 5 mL vial, containing a platinum coated resin as catalysator in the headspace above the sample. The sample was then equilibrated with 10% H_2 in helium at 30 °C for 2h. The equilibrated sample gas was cleaned on a GC column at 80 °C and then loaded into the mass spectrometer. The applied H^{3+} correction factor was 3.76. After this, the same sample was equilibrated with 5% CO_2 in He at 30 °C for 6 hours, the CO_2 separated on a GC column at 100 °C and then introduced into the mass spectrometer.

Stable isotope abundances are reported in delta (δ) values as the deviations from conventional standards in parts per mil (‰) from the following equation:

$$\delta X\ (‰) = [(R_{sample}/R_{standard} - 1) \times 1000]$$

where X = ^{2}H or ^{18}O and R = the ratio $^{1}H/^{2}H$ or $^{16}O/^{18}O$. Both values are reported respective of the Standard mean Ocean Water (SMOW) scale.

Each batch of 60 samples included at least 15 standards, 3 at natural abundance and 12 at 4 distinct levels of enrichment. Laboratory standards at natural abundance levels were calibrated against SMOW and SLAP and the enriched standards against IAEA-607. Precision was 3.0 $^{0}/_{00}$ for hydrogen and 0.15 $^{0}/_{00}$ for oxygen isotopic values, respectively.

3.2.3. Energy Expenditure and Water Turnover calculations

CO_2 production was estimated by using the single pool model described by Speakman (1997, equation 7.17). Initial isotope dilution spaces were calculated by the plateau method and used in millilitres assuming a value of 18.020 as body water molecular weight. Final dilution spaces were calculated by the percentage method. The average between initial and final dilution spaces was used in the final calculations of FMR. The production of CO_2 was converted into energy expenditure by assuming a Respiratory Quotient (RQ) of 0.8 using the Weir equation (1949). Predicted field metabolic rates for mammals of equal body mass were calculated by using equation 3 from Nagy (1999).

3.2.4. Statistical analysis

Multi-model inference with an information-theoretic approach (Burnham & Anderson 2003) was employed to contrast the adequacy of four working hypotheses (the candidate models) about FMR and water turnover. Given our low recapture rate, which resulted in a

small sample size (N = 7), we kept to a minimum the number of candidate models (4) to minimize the likelihood of spurious results (Burnham & Anderson 2003). Therefore, we evaluated as explanatory variables only the single effects of body weight, body condition, disease status and total tumour volume. Sex was not included in the model formulations due to its correlation with disease status (all healthy individuals were males, while diseased individuals were females). All analyses were carried out in R version 3.4.1.

3.3. Results

A total of 7 individual devils (4 males and 3 females) including 5 adults and 2 juveniles were recaptured and then their metabolic rates measured during the field-experiment (Table 3-1). Male individuals presented an average body mass of 6,733 ± 763 grams, while females presented an average mass of 5,850 ± 239 grams. DFTD status of the sampled individuals presented a sex bias with all the infected individuals being females. Cancer burden measured by total tumour volume in females ranged between 0, in the healthy individual, and 299,758 mm^3. The reproductive status of females also varied between females lactating with two and three active teats, and no lactation (no reproductively active) in the case of the juvenile individual and the female presenting the largest tumour burden.

Overall, FMR was higher in the female individuals with DFTD, relative to the males without DFTD (Table 3-1, Figure 3-1). The model selection indicated that from the 4 candidate models tested, there was no single best-fit (Table 2). The model containing total tumour volume was the most supported (Akaike weight of 0.44) and an $R^2 = 0.25$. The second and third ranked models included disease status and body condition as predictors, respectively, and showed Akaike weights of 0.29 and 0.18, respectively. The least supported model included body weight as predictor and accounted for only 8% of the AIC weights and a $R^2 \approx 0$.

Consistent with FMR, water turnover was higher in diseased than healthy individuals (Table 3-1). The model selection indicated that two models presented with an equivalent goodness-of-fit (Table 3-2). The model for disease status had the most support (Akaike weight of 0.68, and $R^2 = 0.62$), followed by the model containing total tumour volume (Akaike weight of 0.29, and $R^2 = 0.51$). Models including body weight and body condition as

predictors presented limited support with delta AIC values of 7.03 and 8.08 and Akaike weights of 0.02 and 0.01, respectively and $R^2 \approx 0$.

Table 3-1 Individual information, water turnover, and metabolic demand of wild Tasmanian devils.

ID	Sex	Age	DFTD mm3	Litter size	Body Mass (gr)	Body Water (%)	Water turnover ml kg-1 d-1		Metabolic and food demand empirical			FMR Predicted
							In	Out	lt CO_2 kg^{-1} d^{-1}	kJ kg^{-1} d^{-1}	Food required (FM d^{-1})	kJ kg^{-1} d^{-1}
1	M	J	0	NA	6100	62	91.32	119.85	275.30	303.75	380.87	320.09
2	M	A	0	NA	6500	64	55.86	62.05	341.98	380.78	532.25	280.38
3	M	A	0	NA	8200	68	73.94	73.98	356.08	374.46	631.17	337.39
4	F	J	0	0	5500	64	86.13	80.18	401.43	414.47	468.58	301.12
5	F	A	12,06	3	5500	78	253.96	265.33	389.62	446.98	505.34	295.64
6	F	A	110,58	2	5900	72	130.53	138.83	476.52	543.59	659.25	264.81
7	F	A	299,75	0	6100	73	164.72	164.66	1118.15	1175.87	1474.41	283.35

Table 03-2 Model structure and selection summary for field metabolic rate and water turnover in wild Tasmanian devils.

Model terms		*AIC*	*ΔAIC*	*AICw*	*R^2*
Field Metabolic Rate					
FMR = 505.08 + (51.45 ± 29.57)	*x log_{10}(Total Tumour Volume)*	109.76	0	0.44	0.25
FMR = 418.30 + (315.70 ± 217)	*x (Disease Status yes/no)*	110.60	0.84	0.29	0.15
FMR = 1800 – (1312 ± 1177)	*x (Body Condition)*	111.52	1.76	0.18	0.03
FMR = 423 + (223.8 ± 0.32)	*x (Body weight kg)*	113.07	3.31	0.08	≈ 0
Water Turnover					
WT = 76.81 + (106.26 ± 32.16)	*x (Disease Status yes/no)*	83.87	0	0.68	0.62
WT = 108.81 + (14.35 ± 5.26)	*x log_{10}(Total Tumour Volume)*	85.61	1.74	0.29	0.51
WT = 476.09 – (0.06 ± 0.06)	*x (Body Weight kg)*	90.91	7.03	0.02	≈ 0
WT = 161.44 – (41.16 ± 290.83)	*x (Body Condition)*	91.95	8.08	0.01	≈ 0

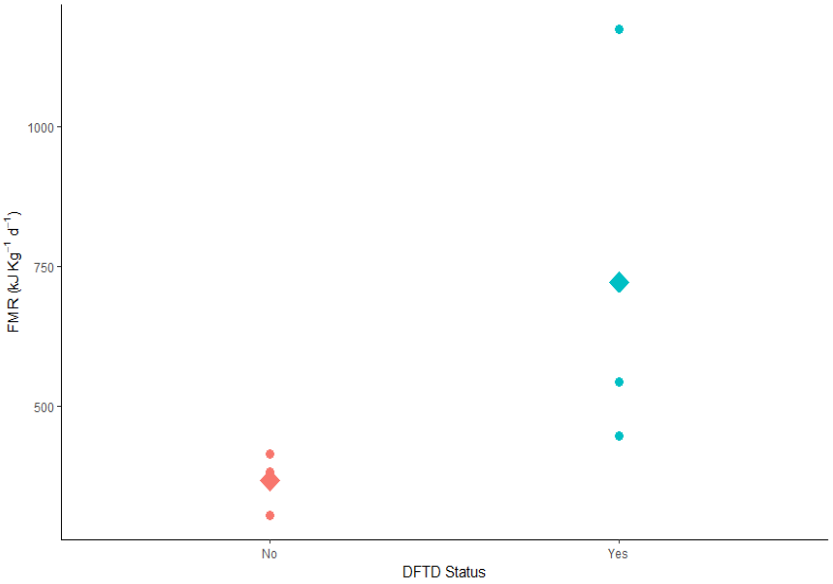

Figure 3-1 Metabolic demand in healthy and diseased Tasmanian devils. Large squares represent the mean for each group.

Figure 3-2 Tasmanian devil infected with DFTD active during day time.

3.4.Discussion

Energy balance is a major driver of the ecology and evolution of organisms and it is a critical component of the pathophysiology of diseases at the individual level. We present the first evidence of enhanced field metabolic rates and water turnover in wild Tasmanian devils infected by a transmissible cancer, Devil Facial Tumour Disease. Infection status and cancer progression, measured either as diseased or healthy individuals or total tumour volume, showed consistent relationship with enhanced field metabolic demand and water turnover of the harbouring host. The metabolic demand in infected individuals may directly compromise fitness components of the host such as survival and reproduction. Further studies with larger sample sizes of infected individuals should examine whether some hosts may be able to cope with the increased metabolic expenditure at tumour burdens below a threshold after which energy reallocation comes at a cost to other organic functions. Our results enhance understanding of cancer progression at the individual level and reveal how metabolic demand shapes the pathophysiology of DFTD.

Our results suggest that DFTD infection and progression may affect the field metabolic rate and water turnover of infected Tasmanian devils. Although, it is important to highlight the limitations of our study derived from the small sample size and the sex bias in the infection status (all infected individuals were females), our data supports an enhanced metabolic demand derived from cancer progression. Overall, all infected individuals presented higher field metabolic rates and water turnover than healthy ones. In the case of water turnover, infected individuals presented values of at least 60% higher than the mean of healthy individuals (mean water in = 76.81 ml kg^{-1}) regardless litter size. The highest value of water turnover was recorded in an infected female with a small tumour volume, in which case it may reflect the demand from the litter. In the case of the infected individual without pouch young the water turnover presented values higher than in healthy individuals but intermediate in relation to the other two infected females. These findings might support that tumour burden may drive water turnover to levels similar to a medium size litter. In the case of field metabolic rate (FMR), infected individuals presented higher values than healthy individuals. The maximum FMR recorded was in an infected female with the maximum recorded tumour volume (~300 ml) which did not present dependent pups, however it presented an FMR equivalent to more than twice the one recorded for the other infected individuals. This finding suggest that large tumours in late stages of progression may represent a metabolic demand

which may surplus the one derived from reproduction in female Tasmanian devils. Thus, one potential strategy of females to cope with the cancer when infected during pregnancy or lactation may be by adjusting litter size (Ruiz-Araven*a et al*, 2018).

Field Metabolic Rates operates on both host and pathogen at the time of infection imposing simultaneous restrictions on their performance and overall biology. We predict that metabolic rate may act as a pacemaker of the devil-cancer ecology. Metabolic rates are expected to act on the extended phenotype of the interaction between host-pathogen, favouring efficient strategies of joint energy exploitation of both, devil and tumour. From the devil perspective, though only measurable for one individual, the fitness costs of this chronic disease – host fitness association was evident in one infected female in this study, which was of reproductive age but with no pouch young and presented the highest metabolic demand and largest tumour volume. From the DFTD perspective, the enhanced FMR induced by the growing tumour may compromise the cancer fitness by two means: increasing cell death or decreasing transmission rate. In the first case, as DFTD is a chronic condition in which host energy demand increases over time as a consequence of the uncontrolled replication of tumour tissues, the enhanced metabolic demand increases the chances of tumour cells dying of necrosis due to 'metabolic catastrophe' (demand is higher than the energy available) (Jin *et al.*, 2007). Alternatively, since DFTD fitness is linked to its capacity to be transmitted to a new hosts by direct contact, the higher FMR of infected hosts may alter the transmission likelihood by inducing behavioural changes in infected individuals (Funk *et al.*, 2010; Ezenwa *et al.*, 2016). For instance, infected hosts may change their foraging patterns to compensate for the high demand of nutrients (i.e. extending foraging hours or decreasing searching periods) (Bohn *et al.*, 2016; Martin *et al.*, 2018), potentially affecting the patterns of social interactions. This is supported by anecdotal records of sightings of individuals in advance stages of infections during daytime. Other changes in behaviour may derived from the progressive weakness experienced by infected individuals as cancer burden increases (Ruiz-Aravena *et al.*, 2018) which may reduce their ability to compete for food or reproduction and therefore affect their potential contacts with other individuals in the population (Hamede *et al.*, 2008; Hamilton *et al.*, 2019). Further field studies with larger sample sizes will prove valuable to assess how strongly the metabolic demand in infected devils translates into epidemiological patterns of DFTD in the wild by affecting transmission, tolerance and resistance to the cancer.

3.5. References

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Chapter 4

Impact of DFTD progression on
body condition of wild Tasmanian devils

CHAPTER 4. IMPACT OF DFTD PROGRESSION ON BODY CONDITION OF WILD TASMANIAN DEVILS

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4.1. Introduction

Host and pathogen populations are entangled in ongoing conflicts of interest which result in complex eco-evolutionary dynamics. Pathogens exploit host resources to replicate and transmit, a process that imposes fitness costs on the host (Finnerty *et al.*, 2017; Simpson *et al.*, 2016). Hosts express two distinct defence strategies to cope with infection and decrease the effects of pathogen burden: resistance and tolerance (Råberg *et al.*, 2009; Simms and Triplett, 1994). Resistance is a mechanism by which hosts directly attack the pathogen, reducing pathogen burden and therefore negatively affecting pathogen fitness. Tolerance, on the other hand, is a mechanism by which the host can buffer the negative impact of infection on its health without reducing the pathogen fitness (Råberg *et al.*, 2009; Schneider and Ayres, 2008; Svensson and Råberg, 2010). There are epidemiological and ecological costs and benefits for a host investing in either of these energetically expensive defence mechanisms (Knutie *et al.*, 2017; Restif and Koella, 2004), and hosts tend to display the strategy, or combination of both, that provides the best cost/benefit balance (Best *et al.*, 2014; Råberg *et al.*, 2007; Read *et.al.*, 2008; Roy and Kirchner, 2000). Tolerance, which translates to host ability to cope with infections is proposed to be more beneficial to hosts than resistance when the risk of becoming infected is high as a result of high disease prevalence (Best *et al.*, 2014; Roy and Kirchner, 2000). This strategy may increase the infectious period in hosts, and therefore may also benefit the pathogen in the long term by increasing pathogen prevalence,

and with it the benefits of hosts carrying genes able to buffer the impacts of infection in the population (Roy and Kirchner, 2000). On the other side, resistance would decrease pathogen prevalence in the host population, and as a result the benefit for resistant genotypes will decrease in the long-term. The reduced prevalence, at a cost for pathogen fitness, would act as a selective force in favour of pathogen genotypes expressing strategies to counter balance host resistance. Thus, the evolution of resistance will drive the host-pathogen evolution to an arms race or red queen dynamic, while tolerance may lead the system towards a stable evolutionary dynamic and eventually coexistence (Read *et al.*, 2008).

The effect of disease progression on host health can be illustrated graphically by plotting the reaction norm of host health as predicted by pathogen burden (Råberg *et al.*, 2009b; Simms, 2000). The slope of the resultant curve indicates the level of host tolerance to infection, with a horizontal (zero) slope indicative of a completely tolerant individual and a negative slope, a potentially less tolerant one. Hence, the steeper the slope, the greater the impacts of pathogen burden on host health. The resultant slopes provide information about the overall impact of the disease on a group of hosts, allowing comparisons between groups or populations, (e.g. sexes, age classes, genotypes, sites). The intercept of the graph (called vigour) may indicate the level of health or fitness of the host population when there is zero pathogen burden (Råberg *et al.*, 2009).

Here, we investigate the effect of a recently emerged infectious disease on body condition of wild Tasmanian devils. By using body condition as a proxy for health, we assess the shape and magnitude of the reaction norm of host health to the progression of devil facial tumour disease (DFTD), a transmissible cancer. DFTD is characterised by solid tumours growing on the facial, oral and neck region of Tasmanian devils (*Sarcophilus harrisii*) (R Loh *et al.*, 2006). The infectious agent is live tumour cells transmitted by direct inoculation when susceptible and infected individuals bite each other (Pearse and Swift, 2006). In almost 100% of cases, the host dies within 12 months after the clinical presentation of a solid tumour (McCallum *et al.*, 2007). This high mortality has diminished wild Tasmanian devil populations locally by more than 90% (McCallum *et al.*, 2007), with a decline in median density of 77% across 80% of its distributional range (Lazenby *et al.*, 2018). Disease-induced extinction of devils in the wild was raised as a genuine concern, based on the frequency-dependent transmission mode of DFTD (McCallum *et al.*, 2009), however, the species has persisted in the long term in infected areas, though in low numbers. Evolution in the

interacting host and pathogen populations may be the cause of the persistence of devils in the wild. Changes in tumour variants (Murchison *et al.*, 2012; Pearse *et al.*, 2012) and in the epidemiology and population impacts associated with tumour lineage replacement (Hamede *et al.*, 2015) have been described, as well as spontaneous tumour regressions (Pye *et al.*, 2016). From the host side, immune responses against cancer, and rapid changes in the genome in populations related to disease arrival have been reported (Epstein *et al.*, 2016). Epstein et al. (2016) identified seven genes in two regions of the genome that may be under selection. In a second study, analysing the data generated by Epstein et al. (2016), the authors identified 97 signatures of selection potentially associated with cancer and behaviour related phenotypes in devils (Hubert *et al.*, 2018). Finally, a recent study has found associations between SNPs and DFTD-related phenotypes (i.e. survival to infection, age of first infection) in devils, reporting differences between males and females, with much more phenotypic variation being explained by SNPs in females than male devils (Margres *et al.*, 2018). All of these genome-focused findings suggest an ongoing evolutionary process between devils and DFTD, in which this newly emerged pathogen represents an intense selective force on host populations (Hubert *et al.*, 2018). Evaluation of phenotypic changes in tolerance and resistance which may shed light on functional interactions between cancer and host is limited. In this context, the Tasmanian devil and its transmissible cancer provides a case study system to examine how hosts and pathogens interact at both individual and population levels in the early stages of their ecological and evolutionary history.

4.2. Methods

4.2.1. Study sites and sampling

Two study sites of 25 km^2 were established in northwest Tasmania, Australia, in the localities of West Takone (379369 E / 6440624 S) and Wilmot (426417 E / 5414182 S). The devil populations at the two sites were each sampled ten times, at three-month intervals, between February 2015 and August 2017 (February 2016 was not conducted) to address seasonal and physiological changes due to reproduction. Each field session consisted of 10 continuous trapping days deploying 40 custom-built carnivore traps. On the first capture, each individual devil was permanently marked with a subcutaneous microchip, and sex and age (using tooth wear, accurate to 3 of 5 years lifespan) were determined. Body mass and

head width (zygomatic width) were recorded as morphometric proxies. For individuals presenting with clinical signs of infection with DFTD, a photo-identification of individual tumours was captured, and the maximum length, width and depth of tumours measured, volume being calculated later using the ellipsoid volume formula. Infected devils often presented with more than one tumour in various locations on the body at the same time, therefore total tumour volume was calculated by summing the volumes of all tumours present in a host at the time of observation. Total tumour mass was calculated assuming a density of 1.1grams per ml of volume as described for soft tissues of similar composition than DFTD cells (Woodard and White, 1986).

4.2.2. Body condition

We applied an index based on the relationship between body weight and body size, that is widely used for measuring and comparing body condition in individual mammals (Stevenson and Woods, 2006). The body condition index was calculated by dividing the body weight (body weight minus tumour weight) for each individual by the body weight expected from the linear regression of body weight with head width (a precise measure of animal size because it is measured across bone - the jugal arches - with minimal overlying tissue), both log-transformed, for the population. The resulting index represented a proportion between actual and expected weight. Study site was not included as a factor in the regression models constructed to build a generic allometric relationship for devils. The regression equation was built using observations only from clinically healthy individuals (no macroscopically detected tumours). This index provided a biologically meaningful indicator of how much above or below the expected body mass an individual was according to its body size. Independent linear regressions were fitted for males and females to take account of sex differences in the relationship between body mass and body size. The subsequent analyses used the body condition index according to the respective equation for males and females.

4.2.3. Effect of DFTD progression on body condition

The effect of disease progression on body condition was assessed by using Generalized Additive Mixed Models to account for potential non-linear effects of cancer progression on body condition index. We used two proxies of pathogen burden or disease progression as predictor variables, in this case: i) total tumour volume (Tvol), which was the arithmetic sum of the individual volume of all observable tumours present on a host at the

time of measurement; and ii) an estimate of the ratio between total tumour weight and body mass (T/B). The latter index proportionated an indicator of pathogen burden in relation to host body mass. Preliminary analyses were conducted to assess potential differences in maximum tumour burden recorded in males and females (details in Supplementary Material). Season and Julian day were also included as non-linear variables in model formulations to consider for natural changes in body condition that devils experience through the year because of life history events such as reproductive effort. Thus, season and Julian day were transformed to circular variables using its cosine transformation in model formulations. We included individual as a random effect in the model formulations to account for non-independent observations because of the repeated measures from the same individual devils at various times. Field trips were conducted in summer - February, autumn - May, winter - August and spring - November of each year. We formulated a total of eight models, accounting for all single and pairwise combinations of the four predictor variables, avoiding building models that included correlated variables: tumour volume vs. tumour body weight ratio, and season vs. Julian day. We applied a multi-model selection approach based on Akaike Information Criteria (AIC) to rank the models (Burnham and Anderson, 2002). We calculated relative importance of the predictor variables by adding the model weights for all models including each variable. This analysis was run using only adult individuals (>18 months old) with clinical infection. Finally, we included the statistical significance for the smooth terms included in the best fit models to assess for sex differences influencing the effects of the other explanatory variables. All statistical analyses were conducted in R software version 3.4.1.

4.3.Results

A total of 625 capture events and observations were recorded during the study period. The total data set included 298 individual Tasmanian devils (158 females and 140 males). A total of 462 observations of healthy individuals were used to fit the allometric curve to calculate the body condition index with 237 and 225 observations for female and male devils, respectively. A total of 96 observations of infected females and 67 of infected males were recorded, which comprised the dataset analysed to test the effect of disease progression on body condition.

4.3.1. Effects of variables on body condition

The model with most support (97.4% of AIC weights) included T/B and its interaction with host sex and explained 49.8% of the null deviance (Table 4-1). All others seven model formulations presented little support, however they evidenced significant statistical effects. Tvol alone and the interaction of Tvol with host sex, showed an overall significant effect on host body condition ($p = 0.039$, and 0.023, respectively), and the models explained 24.4 and 29.3% of the null deviance. Season and Julian day showed also limited explanatory capacity in the analyses ($AICw \approx 0$). The variable importance calculation showed that T/B is the most important variable in explaining the body condition of infected devils (99.95%), followed by host sex (97.4%).

4.3.2. Sex-specific differences in tolerance to DFTD

The shape and magnitude of change in body condition differed between sexes (Figure 1). Disease progression presented a significant stronger effect on males than in females ($p = 0.001$). This pattern was consistent across the two measures of disease progression Tvol and T/B (Figure 4-1a – d). T/B showed an almost linear (negative) effect on body condition of males, decreasing body condition by approximately 25% between the early and late stages of the disease (Figure 4-1b). In females, T/B presented a similar negative trend with an overall decrease in body condition of approximately <5% at T/B of more than double the experienced by males (Figure 4-1a). Despite the low support presented by the models including Tvol, a significant effect of Tvol and its interaction with sex was detected ($p = 0.039$ and 0.023, respectively). both sexes presented with similar maximum values of total tumour volume. While the curve for females shows that they may be able to almost maintain their body condition with disease progression (Figure 4-1c), in males the effect follows a non-linear shape with a slight capacity to maintain their body condition in initial stages of infection (Tvol < 3) followed by a decline of approximately 15% between middle and large tumour volumes (Figure 4-1d).

Table 4-1 Results from the Mixed effects Generalized Additive Models analysing the effects of seasonality and cancer progression on body condition of Tasmanian devils infected with DFTD.

Intercept	Model terms	p-value[+]	AIC	ΔAIC	AICw (%)	D^2
0.003	s_1(T/B, df = 0.744)	0.047*	-233.585	0	97.40	49.8
	s_2(T/B:Female, df ≈ 0)	0.494				
	s_3(T/B:Male, df = 0.921)	0.001**				
0.004	s_1(T/B, df = 2.267)	-	-226.300	7.285	2.55	23.3
0.003	s_1 (Season, df = 1.316)	-	-217.742	15.843	0.03	38.7
0.004	s_1 (Tvol, df = 1.848)	-	-213.531	20.054	≈ 0	24.4
0.003	s_1 (Day, df ≈ 0)		-212.473	21.112	≈ 0	32.2
	s_2 (Day:Female, df ≈ 0)					
	s_3 (Day:Male, df = 1.741)	-				
0.006	s_1 (Tvol, df = 1.145)		-211.534	22.051	≈ 0	29.3
	s_2 (Tvol:Female, df ≈ 0)					
	s_3 (Tvol:Male, df = 0.796)	-				
0.003	s_1 (Day, df ≈ 0)	-	-209.092	24.493	≈ 0	28.8
	s_1 (Season, df = 1.323)	-	-205.421	28.164	≈ 0	30.9
0.004	s_2 (Season:Female, df ≈ 0)					
	s_3 (Season:Male, df ≈ 0)					

T/B = Tumour weight – host body weight ratio; Tvol = Total tumour volume; Day = Julian day of the year; Season = season of the year. Significance levels of the smooth terms for each model are presented (*p-value*).

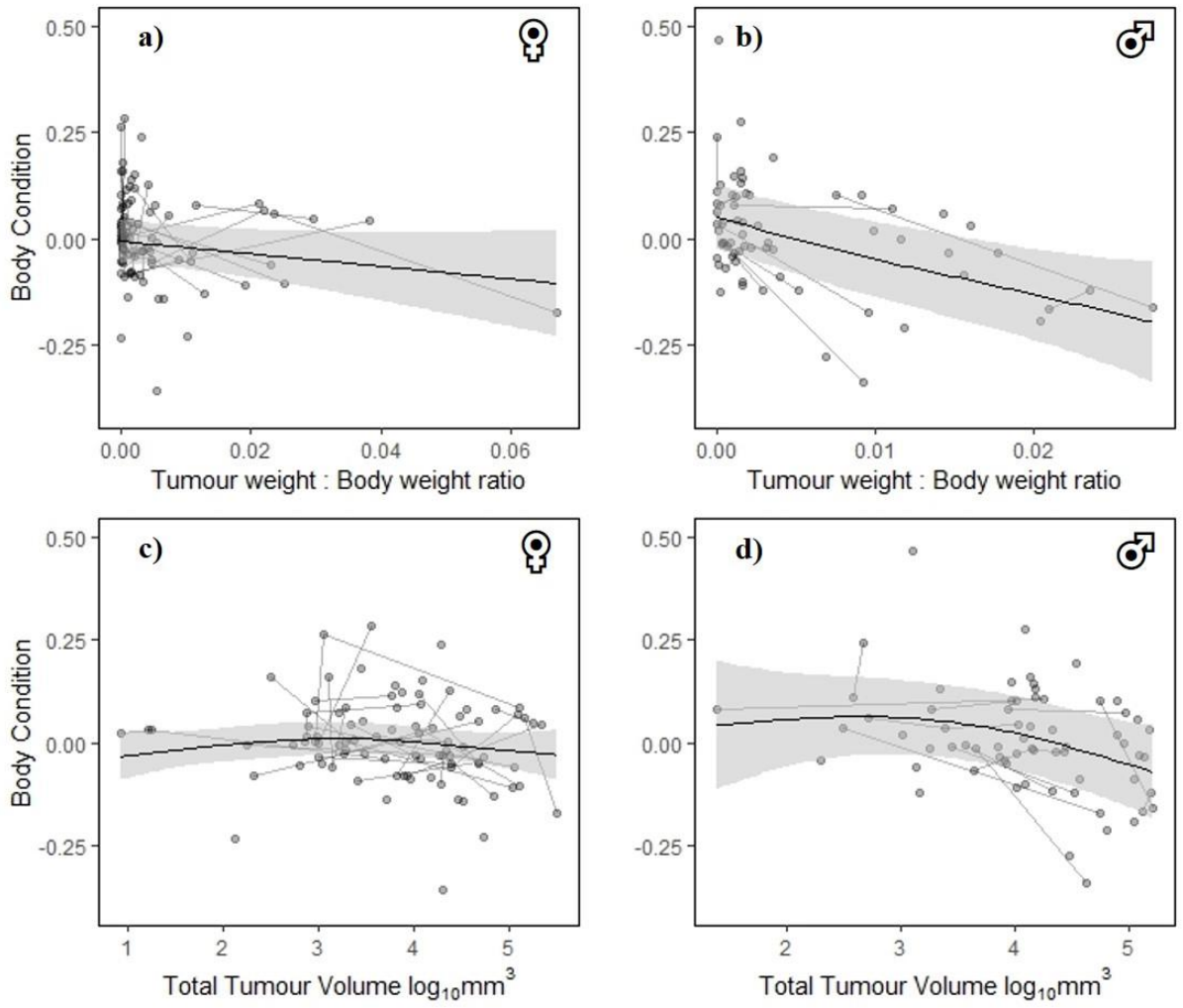

Figure 4-1 Effect of cancer burden on body condition of female and male Tasmanian devils. (a,b) Reaction norms of body condition by tumour-body weight ratio for (a) females and (b) males. (c,d) Reaction norms of body condition by total tumour volume for (c) females and (d) males

4.4. Discussion

We present an evaluation of the effects of disease progression on host body condition in wild populations using a field-based approach. Our study is the first evaluation of tolerance to infection at the population level in a recently emerged transmissible cancer in wild Tasmanian devils. Disease state, as measured by cancer burden (tumour - body weight ratio, and total tumour volume), has an overall negative effect on body condition of hosts infected with DFTD, with a much greater magnitude of the effect on male than on female devils, suggesting a sex bias in tolerance to this transmissible cancer. The sex differences in the shape of the reaction norm of body condition as disease progresses suggest that proximate strategies to cope with infection and their consequent evolution should differ between male and female hosts. This could result in differential fitness of infected males and females, with sex-linked survival and fecundity rates as energy reserves deplete.

Our results reveal sex-related differences in the effects of cancer progression on Tasmanian devils. Tumour to body weight ratio (T/B) and total tumour volume (Tvol) have an overall negative effect on the body condition of infected individuals, with a much stronger negative effect in males than females (Figure 4-1). These sex differences are demonstrated by a significant interaction between sex and disease progression in the model formulations. Tumour to body weight ratio presents a significant negative linear effect on body condition of males, which may suffer declines of approximately 25% of body condition when cancer burden reaches around 3% of the body weight. This is of a similar magnitude to the reproductive cost in loss of body condition experienced by non-infected males during the mating season (Jones *et al.*, 2008). In contrast, the negative effect of T/B on females is much less, with body condition declining by approximately <5% when tumour weight reaches 6% of host body mass. Models including total tumour volume have less support than for T/B. Tumour volume in males shows a non-linear effect on body condition with a marginal increase at initial stages of the infection, a detrimental effect at around tumour volume of 3 ($\log_{10}$ scale of mm^3) and an overall loss of approximately 15% in body condition. In contrast, body condition of females appears to be less influenced by tumour volume than by T/B, although condition trends down at larger tumour volumes.

There is an apparent overall threshold in tumour volume (of 3; $\log_{10}$ scale of mm^3), after which body condition declines in males, and slightly in females. This finding is

consistent with theoretical findings that infected individuals potentially have higher survival than non-infected ones at low tumour volumes, but lower survival than non-infected individuals when tumour burden is higher than 100 ml (Wells *et al.*, 2017). This pattern may suggest a threshold for cancer burden, at which infected individuals may be able to completely tolerate the infection. This level of cancer burden may also represent a physiological and metabolic landmark indicating the moment at which the disease becomes a significant compromise to the fitness of the host. (Louie *et al.*, 2016).

Body condition in wild populations is expected to vary according to the ecological context in which organisms inhabit, and to follow seasonal patterns of availability of resources and reproductive investment (Simard *et al.*, 2014). Tasmanian devils are seasonal breeders, with the mating season taking place in late summer and early autumn (Keeley *et al.*, 2012b, 2017). This pattern allows juveniles to be weaned during mid and late spring. The costs of reproduction are expected to differ between sexes in magnitude and timing. Energetic demands are expected to peak during the mating season in males (Hesterman and Jones, 2009), and during the second half of lactation in females, starting in winter when they are carrying large pouch young up until early-summer when the denned young are weaned (Hesterman *et al.*, 2008a, 2008b). The limited support for models that include seasonal variables (season and Julian day) suggests that cancer progression may over-rule the effect of seasonality on body condition of infected individuals. As cancer progresses, its growth and/or secondary complications (Loh *et al.*, 2006; Peck *et al.*, 2016) may increase the energetic demand of infected individuals with a stronger effect on the resulting body condition than that driven by seasonal patterns of reproduction and food availability. The lack of a seasonal effect in females may reflect plasticity in their ability to manipulate reproductive costs by reducing provisioning and number of young in a litter according to environmental conditions, or a response to increased resource availability as populations decline well below carrying capacity due to DFTD-induced mortality. The latter effect has been proposed as the proximal cause of the increased proportion of precocial breeders in diseased populations (Jones *et al.*, 2008).

The differences in tolerance to cancer progression between males and females may select for changes in life history strategies in the host and could also alter the selective environment for the tumours. As the impact of disease progression on host health differs between the sexes, the environmental and ecological context for the cancer will also differ

based on whether the tumour is carried by a male or a female devil. Body condition is directly linked to survival and reproductive success in natural populations (Budischak *et al.*, 2017; Wauters and Dhondt, 1995) and the ability to tolerate infection, and maintain body condition as pathogen burden increases, is therefore a trait potentially under selection. There are two main influences from DFTD on life history plasticity in both sexes of devils. The first is high mortality following the first mating season (Lachish *et al.*, 2009), when much of transmission is thought to occur through biting behaviour (Hamede *et al.*, 2008, 2013), placing selection pressure on terminal investment, expressed as early or precocial breeding in the first year of independent life (Jones *et al.*, 2008). The second is the increased growth rates in juveniles, enabled by improved nutrition following severe population decline, that can facilitate early sexual maturity and precocial breeding (Lachish *et al.*, 2009). Reproduction and mating systems place different energetic demands and selection pressures on males and females and so they may respond differently, in the flexibility they have in energy allocation and in life history. Increased precocial breeding in female devils (Jones *et al.*, 2008; Lachish *et al.*, 2009) extends the reproductive season for males, which can mate multiple times since their reproductive investment is not influenced by seasonality (Keeley *et al.*, 2012a). This may allow infected males, which already have compromised energy resources from DFTD that are equivalent to mating season effort, to spread their reproductive effort over a longer time period instead of the intense, short mating season that occurs in healthy populations (Hesterman and Jones, 2009). Females, on the other hand, are constrained to one litter per year but flexibility in reproductive effort may be available through manipulating sex ratio, litter size and maternal investment in that litter (Lachish *et al.*, 2009).

Phenotypic variation among individuals in natural populations, that has a genetic basis, is the basis upon which natural selection acts. The genetic basis of and phenotypic variability in tolerance and resistance to infection has been documented in wild populations and demonstrated to be under selective force (Anaya-Rojas *et al.*, 2016; Hayward *et al.*, 2014b, 2014a). Tolerance and resistance differ in their associated costs. Resistance is potentially costlier to the host than tolerance, perhaps as a consequence of the level of the specificity of the underlying mechanisms (e.g. production of antibodies that confer resistance) (Moret and Schmid-Hempel, 2000). In the devil—DFTD system, fitness costs associated with a host immune response (resistance) are indicated by tumour strategies which allow them to evade immune recognition, such as down-regulation of MHC expression (Siddle *et al.*, 2013). Evidence of resistance against DFTD is sparse. Despite devils having a

fully competent immune system and the ability to mount immune responses to DFTD when induced (Pye *et al.*, 2018; Tovar *et al.*, 2017), the incidence of spontaneous tumour regression in wild populations is currently low. However, some wild devils whose tumours have regressed spontaneously have shown immune responses to DFTD (Pye *et al.*, 2016) and genome-wide association analysis of these devils reveals two genomic regions potentially associated with the ability to spontaneously recover (Wright *et al.*, 2017). With tolerance, the costs associated with proximal mechanisms may be lower than for resistance. Generic strategies, such as energy reallocation do not require specific biochemical "machinery" to be put in action. This could potentially result in the emergence of tolerance earlier than resistance in the evolutionary time of new host-pathogen interactions, as in the devil-DFTD system. (Lachish *et al.*, 2009; Trivers and Willard, 1973). It is possible that the changes in tumour lineages (Hamede *et al.*, 2015), epidemiology (Hamede *et al.*, 2011), and sex differences in individual survival (Margres *et al.*, 2018) in infected populations are a consequence of distinct levels of effects of DFTD infection, that are related to sex and body mass of the host, where males are larger than females. This may result in tumour-male and tumour-female systems having a different influence on infective times, behaviour and therefore transmission dynamics.

Cancer has been considered an individual condition with very limited potential to act as a force in natural selection, and therefore in evolution. However, an increasing number of studies have shown that oncogenic phenomena play an important role in the ecology and evolution of their hosts and ecosystems (Thomas *et al.*, 2017; Vittecoq *et al.*, 2013). Transmissible cancers, of which only eight naturally occurring cases are known to science, behave as infectious agents, but the pathology experienced by hosts are the hallmarks of cancer such as uncontrolled cellular replication within tumours, tissue invasion, immune evasion and metastasis (Hanahan and Weinberg, 2011, 2000; Ujvari *et al.*, 2016). In this context, studying how different host genotypes respond to infection by and progression of clonal tumours will help to understand cancer biology in an evolutionary context, which may expand our understanding of the internal battle between cancerous and healthy host tissues (Frampton *et al.*, 2018; Murgia *et al.*, 2006; Siddle and Kaufman, 2013; Tovar *et al.*, 2017; Ujvari *et al.*, 2016; Woods *et al.*, 2007). Cancer risk in humans has been reported to vary with factors such as sex (Rakoff-Nahoum and Medzhitov, 2007), occupation (Bayer *et al.*, 2016; Hadkhale *et al.*, 2016) and concomitant infections (Restif and Koella, 2004). Studies of cancer progression in natural conditions without treatment, which would allow researchers to

explore differences in tolerance among patients, are non-existent for ethical reasons. The applicability of the concept of tolerance, originally developed by plant ecologists and recently incorporated in animal studies, to cancer contexts may provide the framework to understand oncogenesis as an important force in ecological and evolutionary processes. Studying cancer in evolutionary framework and treating cancer as a chronic condition in which tumours act as parasites, may expand thinking around treatment possibilities to target tolerance levels in hosts instead of resistance, which is the current approach (Restif and Koella, 2004).

The devil-DFTD transmissible cancer system is showing signs of evolving at a surprisingly rapid rate. By studying the different dimensions of this process, from ecology and epidemiology to genomic interactions between host and pathogen, we can expand our understanding of cancer biology beyond the specifics of the devil-DFTD system. Evolution is a dynamic process and needs to be better studied and factored into management actions; whether they be conservation of Tasmanian devils in the wild or cancer treatment in humans and domestic animals. Further research to elucidate how much of the individual and sex-related variability in tolerance is explained by genetic or environmental influences, will reveal how natural selection may act in this relatively new host-pathogen system.

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Chapter 5

Immune response relates to infection and progression of cancer in Tasmanian devils

CHAPTER 5. IMMUNE RESPONSE RELATES TO INFECTION AND PROGRESSION OF CANCER IN TASMANIAN DEVILS

5.1. Introduction.

Transmissible cancers, with their ability to spread among individual hosts, are rapidly changing the concept of oncogenic processes being an individual condition with limited consequences for evolution of responses in host populations (Ostrander *et al.*, 2016). Because transmissible cancers act as infectious pathogenic agents (Metzger and Goff, 2016) and can have devastating impacts on host populations (Barber, 2004; Lachish *et al.*, 2007), they have the potential to act as strong selective forces on host populations (Epstein *et al.*, 2016) to develop "cancer-aware" strategies to optimize their fitness. Therefore, natural selection is expected to act in favour of host and pathogen (tumour) variants affecting the dynamics of interaction in the long-term (Brunner *et al.*, 2017; Budischak *et al.*, 2017; Hamede *et al.*, 2015; Lambrechts *et al.*, 2006), which potentially may lead the systems toward coexistence. The oldest cell line on earth is precisely a transmissible cancer, the canine transmissible venereal tumour (cTVT) (Murchison *et al.*, 2014; Rebbeck *et al.*, 2009). cTVT affects domestic dogs and has developed strategies to survive in its host with very limited pathogenicity (Rebbeck *et al.*, 2009). This has favoured transmission and therefore persistence of the disease in the dog populations for as long as 10,000 years, with neither tumours or hosts experiencing strong selection pressure (Murgia *et al.*, 2006). Hosts of transmissible cancers as any other infection may benefit from displaying strategies to control the impact of infection on their fitness such as resistance by mounting an immune response against the tumour tissue and clear it from their organism (Råberg *et al.*, 2009). Here, we assess resistance of Tasmanian devils to the devil facial tumour disease (DFTD), the most recently emerged transmissible cancer, by evaluating the relationship between immune responses against tumours and its effect on infection status and disease progression.

Devil Facial Tumour Disease (DFTD) is the most recently emerged transmissible cancer in nature, and affects the Tasmanian devil, the largest extant carnivorous marsupial. DFTD has caused a dramatic decline of Tasmanian devil populations in the last 25 years leading to their listing as Endangered (IUCN, EPBC Act, state legislation). Since DFTD emerged in a female Tasmanian devil in the north-east of Tasmania in the early 1990s, it has spread across almost 90% of the devil's island distributional range in Tasmania, causing population declines averaging 80% (Lazenby *et al*., 2018), but locally up to 95%. Biting behaviour during social interactions is thought to be the main route of transference and inoculation of tumour cells from infected to naïve hosts (Hamede *et al.*, 2008, 2013). Transmission and establishment of DFTD are facilitated by the incapacity of hosts to reject tumour cells by immune response (Siddle *et al.*, 2013). Two main hypotheses were formulated to explain this incapacity of devils to recognize tumour cells as foreign and mount an immune response: i) Low genetic variability in the MHC-I alleles (Morris *et al.*, 2013; Siddle *et al.*, 2007), and ii) immune escape strategies from the tumour tissues (Siddle *et al.*, 2013; Siddle and Kaufman, 2013). Findings in support of the first hypothesis are contradictory since the devil immune system has been demonstrated to be competent in rejection of allographs (Kreiss *et al.* 2011), however developmental changes in IgM/IgG ratios (Ujvari *et al.*, 2016) and general immune condition (Cheng *et al.*, 2017) may compromise protection against cancer. The second hypothesis is supported by findings of down-regulation of MHC-I molecules in tumour cells. Thus, tumour cells have the capacity of "hiding" MHC-I from their surface which prevents immune recognition and therefore rejection by the host immune system (Caldwell and Siddle, 2017; Siddle *et al.*, 2013).

Regardless of the down-regulation of MHC-I that may compromise or prevent a response against DFTD, some hosts are able to detect the cancer and mount an immune response (Pye *et al.*, 2016). Anti-DFTD responses have been experimentally induced by vaccination of individuals with antigens from tumour cells by using irradiated or sonicated DFTD cells treated with gamma-interferon to induce MHC expression (Tovar *et al.*, 2017). This response has been characterized by an increase in IgG antibodies against DFTD in the immunized devils. In addition, complete rejection of tumours and recovery of individuals can be induced by immune therapy. But it was not until recently that immune responses against DFTD were first detected in natural conditions. Pye *et al.* (2016) first demonstrated the occurrence of antibodies (IgG) against DFTD and cell infiltration of tumours in natural conditions, suggesting that humoral and cell-mediated responses occur at certain moments of

infection by DFTD and therefore the immune escape from tumours may be imperfect. In addition, the authors presented individual cases that may suggest the potential for resistance to the disease and that devils may be able to reject and recover from cancer, however the authors did not perform formal statistical analyses on how the immune response detected may influence disease progression. To shed light on the potential mechanisms mediating tumour remission, Margres et al. (2018) conducted a genome-wide analyses in devils hosting tumours that showed remissions and hosts with tumours that followed the common progression of exponential growth. The results did not show differences between devils with regressions and devils with tumours progressing, suggesting that if regression of tumours is host-driven, it may be associated to differences in gene expression (Margres et al. 2018). Other studies have reported changes in genome regions associated with cancer and immune response after just 4 – 6 generations since disease outbreak (Epstein *et al.*, 2016; Hubert *et al.*, 2018) and potentially "cancer-resistant phenotypes" (Margres *et al.*, 2018). These findings support that DFTD is a strong selective force on devil populations, however there is still a gap in the current characterization of tolerance and resistance that may link the genome-level changes to functional phenotypic responses. So far, cancer remission and recovery of hosts in the wild seem as isolated cases, representing a small proportion of the infected population. This condition may be an artefact of capture efforts and low number of populations sampled at regular intervals that may allow researchers to follow tumour progression, however natural selection is expected to favour evolution of resistance and tolerance to infection in devil populations.

Here, we investigate the host immune response against DFTD and its relationship with disease status and progression in wild Tasmanian devils. We sampled devils and tumours from two wild populations in north-west Tasmania between 2015 and 2018 and evaluate the presence of antibodies (IgG) against DFTD in host serum, and immune cell infiltration in tumour biopsies. We then analyse the data evaluating how antibodies may relate to infection status and progression, and how immune cell infiltration within tumour tissues relates to tumour growth trajectories. We discuss our findings under the context of evolution of resistance to the disease and how what has been evidenced as individual responses may become of relevance at the population level and for the evolution of the devil-DFTD interaction.

5.2. Methods.

5.2.1. Field data and sample collection.

Two study sites of 25 km^2 were established in northwest Tasmania, Australia, in the localities of West Takone (379369 E / 6440624 S) and Wilmot (426417 E / 5414182 S). The devil populations at the two sites were sampled at three-month intervals, between February 2015 and February 2018 (February 2016 was not conducted), to address seasonal and physiological changes associated with reproduction. The resulting data set comprised 12 trapping sessions for the Takone site, and 8 sessions for Wilmot. Each field session consisted of 10 continuous days of trapping in which 40 custom-built carnivore traps were deployed. Upon first capture, each individual devil was permanently marked with a subcutaneous microchip, and sex and age (using tooth wear, accurate to 3 of 5 years lifespan) were determined. Body mass and head width (maximum width across the zygomatic arch), as a linear measurement of size, were recorded. For individuals presenting with clinical signs of infection with DFTD, a photo-identification of individual tumours was captured, and the maximum length, width and depth of tumours measured, volume being calculated later using the formula for ellipsoid volume. Infected devils often presented with more than one tumour in different locations on the body at the same time. In these cases, total tumour volume was calculated by summing the volumes of all tumours present in the host at the time of observation.

5.2.2. Classification of tumour progression.

Tumours were classified as “Progressing” or “Non-progressing” according to the models of DFTD growth rates described by Hamede *et al.* (2017), in which minimum growth was considered to 0.01 *(r)* per day. Thus, tumours with a growth rate between successive field measurements of greater than 0.01 were classified as “Progressing”, while tumours with growth rates below 0.01 were classified as “Non-Progressing”. By using the lowest growth rate of 0.01 described by Hamede *et al.* (2017) as the threshold for classifying tumours, we adopted a conservative approach which minimizes the probability of misclassification. The non-progressing tumours included cases of static growth and cases of tumour remission

(negative growth rate), the latter of which were differentiated for some analyses on tumour progression as “regressing”.

5.2.3. Laboratory analyses

5.2.3.1. Tumour cell culture for antibodies detection by flow cytometry

The DFT1 cell line C5065 was maintained in complete RPMI-10, which was RPMI 1640 (Gibco) supplemented with 10% vol/vol heat inactivated foetal bovine serum FBS (Bovogen Biological), 5mM L-glutamine (Gibco) and 1% vol/vol Antibiotic-Antimycotic (Gibco). All cell lines were grown at 35ºC in a humidified atmosphere of 5% CO_2/95% air. C5065 were stimulated with recombinant devil IFNγ (50ng/ml) for 24 hours to upregulate surface MHC-I expression. MHC-I expression was confirmed by flow cytometry using mouse anti-Tasmanian devil (TD) beta-2 microglobulin (β2m), a component of the MHC-I complex. Untreated and IFNγ treated DFT1 cells were used for screening of serum from wild devils in flow cytometry.

5.2.3.2. Anti-DFTD IgG detection and flow cytometry

All staining and washing was performed in FACS buffer (0.5% BSA, 2.5mM EDTA and 0.02% sodium azide in PBS). DFT1 C5065 cells were aliquoted onto a round bottom 96-well plate (Greiner Bio One) at $2x10^5$ cells/well. Cells were stained in 100µl wild Tasmanian devil (TD) serum (diluted 1:50), incubated for 30 minutes on ice and then washed three times. Cells were then stained in 100µl of 10µg/ml of mouse anti-TD IgG (clone A4/D1; WEHI) for 30 minutes and washed again. Cells were then stained in 50µl of 2µg/ml of Alexa Fluor® 647-conjugated goat anti-mouse IgG (H+L) (Invitrogen) for 30 minutes. Cell were washed again and resuspended in 200µl FACS buffer containing 200ng/ml 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI, Sigma Aldrich). Data acquisition was performed on the FACS Canto II flow cytometer (BD Biosciences) and data was analysed with FlowJo software (FlowJo LLC). Controls included the above cell lines labelled with anti-TD IgG plus the tertiary antibody (no primary control) and serum plus the tertiary antibody (no secondary control), the serum of a captive immunised devil (positive control) and the serum from a wild devil from the DFTD-free insurance population established in Maria Island, Tasmania, with high background staining (internal control). No background fluorescence was observed in the no primary and no secondary controls.

To normalise raw IgG median fluorescence intensity (MFI) data between different days (experiments) that samples were analysed, the following calculations were performed: i) The average IgG MFI of the internal control was calculated from the raw internal control IgG MFI values of each experiment. ii) The raw IgG MFI values of the internal control from each experiment was then divided by the average IgG MFI of the internal control to calculate an internal control IgG MFI division factor. iii) the adjusted IgG MFI for each sample was calculated by dividing the raw IgG MFI by the internal control IgG MFI division factor. The raw values from each experiment day were only divided by the internal control IgG MFI division factor that was calculated from the internal control sample run on the same day to prevent for influences on the data derived from intrinsic variability of the equipment.

5.2.3.3. Histology and Immunohistochemistry

Sixty-two tumour biopsies were selected for microscopical examination to confirm DFTD diagnosis and the level of infiltrations of immune cells associated with cellular immune response. The selected observations included tumours with signs of regression (negative growth rate) and randomly selected tumours classified into two categories: “Progressing” and “Non-Progressing”. The biopsies from devils were screened for DFTD by periaxin staining by immunohistochemistry (IHC) to confirm that they were DFTD tumours. Regressions were confirmed by analysing tissue structure and immune cell infiltration by immune histochemistry (IHC) and histology. Standard hematoxylin and eosin (H&E) and immunohistochemical staining with rabbit anti-human periaxin (HPA001868; Sigma Aldrich), rabbit anti-human CD3 (A0452; Dako), mouse anti-human HLA-DR, alpha-chain (clone TAL.1B5; Dako), mouse IgG1 isotype control (clone MOPC-31C, BD Pharmingen) and rabbit IgG isotype control were performed on 3 mm sections of tumour biopsies fixed in 10% neutral buffered formalin and embedded in paraffin. Assessment of stained biopsies was performed blind by a human pathologist. Visualisation and image acquisition was performed on an Olympus BX-50 light microscope fitted with a Leica DFC320 camera.

5.2.4. Statistical analyses

For all analyses, I used a Bayesian Generalized Linear Mixed Models (GLMM) approach, with Markov Chain Monte Carlo algorithms (MCMC). The chains were sampled by using the Metropolis-Hastings method defined in the MCMCglmm package for R. All

analyses were conducted using R Project for Statistical Computing Version 3.5.1. The specific model formulations associated with each response are detailed below:

5.2.4.1. Disease status and humoral response against DFTD.

The probability of a captured individual devil showing detectable tumours was modelled as a binary response in the MCMCglmm formulation. Predictor variables included host sex, head width as a continuous proxy of physiological age, and median immunofluorescence from the antibodies detection test as a continuous numeric proxy of titres of anti-DFTD antibodies. The interactions of sex and age, and sex and median immunofluorescence as predictor variables were included in the model. Individual devil and experiment identification (see methods of antibody detection) were included as random variables to account for multiple samples of the same individuals. The data included in this analysis comprised 147 observations collected from 49 individual Tasmanian devils from the Takone site. To account for different risks of infection or probability of exposure between the two sites due to differences in DFTD prevalence, only one site was selected for this analysis.

5.2.4.2. Effects of antibodies on tumour progression

Classification of tumours as “Progressing” and “Non-progressing” was modelled as a binary response in a MCMCglmm formulation (Family = categorical). Sex, median immunofluorescence from the antibody detection test and their interactions were included as predictor variables. Since the dataset included tumours measured on more than one occasion in the same host, an interaction term between individual devil and tumour ID, and experiment ID as random variables were included in the model. The data set included 86 observations, including 63 tumours recorded from 28 individual Tasmanian devils.

5.2.4.3. Effect of cellular infiltration on tumour progression

Classification of tumours as “Progressing” and “Non-progressing” was modelled as a binary response in a MCMCglmm formulation (Family = categorical). Predictor variables included the four variables evaluated in the histopathological examination of tumour biopsies: MHC-II intra and peritumour, and CD-3 intra and peritumour. Since the number of samples for which values were obtained for each of the four variables varied, I formulated four independent models for each predictor variable. The interaction between individual devil

and tumour ID (within devil) was included as a random variable. The dataset included a total of 61 observations of 46 tumours recorded from 32 individual Tasmanian devils.

5.3. Results.

5.3.1. Infection status and humoral response against DFTD.

The infection status, modelled as the probability of an individual devil to show signs of DFTD, was influenced by body size and showed trends supporting sex differences in its relationship with IgG (Table 5-1). There was a positive relationship in both sexes between body size and the probability of individual devils to show signs of infection by DFTD (Figure 5-1A). There was no statistical relationship between median fluorescence intensity for IgG and the risk of infection, but the model did show a positive trend in the interaction between sex and MFI and infection status, with male devils presenting a higher probability of showing tumours as MFI increased relative to females (Figure 5-1B).

5.3.2. Tumour progression and immune response.

The effects of immune responses on the classification of tumours as progressing and non-progressing varied among predictors. In the case of humoral response, the model formulation including median fluorescence of IgG detection and sex did not show statistical relationship with the probability of tumours to be classified in either category (Table 5-2). In the case of the histopathological findings, the probability that a tumour progressed was lower as the number of antigen-presenter cells (MHC-II positive) present in the peritumour tissue increased (Figure 5-2A). The number of MHC-II positive cells in the intra-tumour tissue, or CD3 positive cells in the intra and peri-tumour tissue did not show a significant relationship with tumour classification (Table 5-2). In addition, no effect of host sex was detected (Table 5-2).

Table 0-1 Results from the MCMC Generalized Linear Mixed Model for factors that influence the risk of infection by DFTD in Tasmanian devils.

Model term	Post. Mean	Lower CI	Upper CI	Eff. samp	pMCMC
Intercept	-14804.65	-26628.77	-2425.82	589	0.01 *
Sex Male	-8328.56	-29686.31	12306.09	703	0.37
Log_{10}Head Width (mm)	7230.51	936.12	14608.38	605	0.01 *
Log_{10} MFI IgG	18.84	-590.17	599.53	900	0.93
Sex Male : Log_{10}Head Width (mm)	2382.79	-7522.66	12933.07	718	0.60
Sex Male : Log_{10} MFI IgG	1045.92	-236.68	2183.08	900	0.06 .

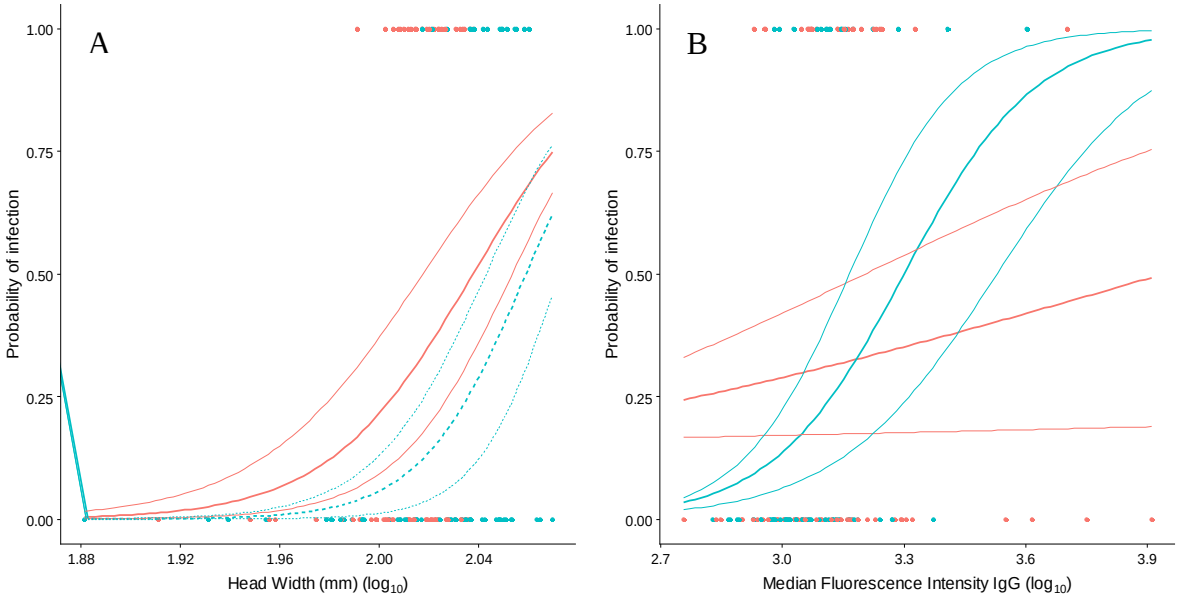

Figure 5-1 Probability of individual devils to present clinical signs of infection by DFTD as a function of body size measured by head width (A) and detection of anti-DFTD IgG (B) for males (light blue) and females (orange lines). The curves depict the mean probability and 95% credible intervals. Points show the individual observations of healthy (0) and diseased (1) individuals.

Table 5-2 Results from the MCMC Generalized Linear Mixed Model for the effect of immune response on tumour classification.

Model	Model term	Post. Mean	Lower CI	Upper CI	Eff. Samp	pMCMC
MFI IgG	Intercept	-3368.5	-9384.5	2059.5	1071	0.185
	Sex Male	-5673.7	-16691.8	3503.2	1131	0.202
	MFI IgG	947.5	-704.0	2817.3	1179	0.231
	Sex Male * MFI IgG	1591.0	-1195.5	4954.6	1262	0.242
MHC-II $^{(+ve)}$ cells peritumour	Intercept	-195.82	-437.03	-2.59	655	0.021*
	Sex Male	-60.24	-424.98	333.68	1107	0.693
	MHC-II $^{(+ve)}$ peritumour	9.07	0.07	18.84	541	0.03*
	Sex Male * MHC-II $^{(+ve)}$ peritumour	7.00	-11.28	24.45	1386	0.35
MHC-II $^{(+ve)}$ cells intra-tumour	Intercept	-275.06	-576.86	-1.59	972	0.01*
	Sex Male	16.16	-445.32	454.79	1977	0.91
	MHC-II $^{(+ve)}$ intra-tumour	5.74	-4.27	17.55	1171	0.23
	Sex Male * MHC-II $^{(+ve)}$ intra-tumour	-0.37	-21.41	19.91	1040	0.94
CD3 $^{(+ve)}$ cells peri-tumour	Intercept	60.27	-270.91	396.70	126	0.62
	Sex Male	-129.17	-707.87	514.83	355	0.60
	CD3 $^{(+ve)}$ peri-tumour	-15.27	-38.42	6.17	108	0.11
	Sex Male * CD3 $^{(+ve)}$ peri-tumour	18.60	-18.22	57.28	187	0.27
CD3 $^{(+ve)}$ cells intra-tumour	Intercept	-60.90	-432.54	312.71	1539	0.73
	Sex Male	-233.78	-838.05	255.84	1675	0.34
	CD3 $^{(+ve)}$ intra-tumour	-5.35	-18.80	8.00	1579	0.38
	Sex Male : CD3 $^{(+ve)}$ intra-tumour	13.02	-5.44	34.91	1342	0.16

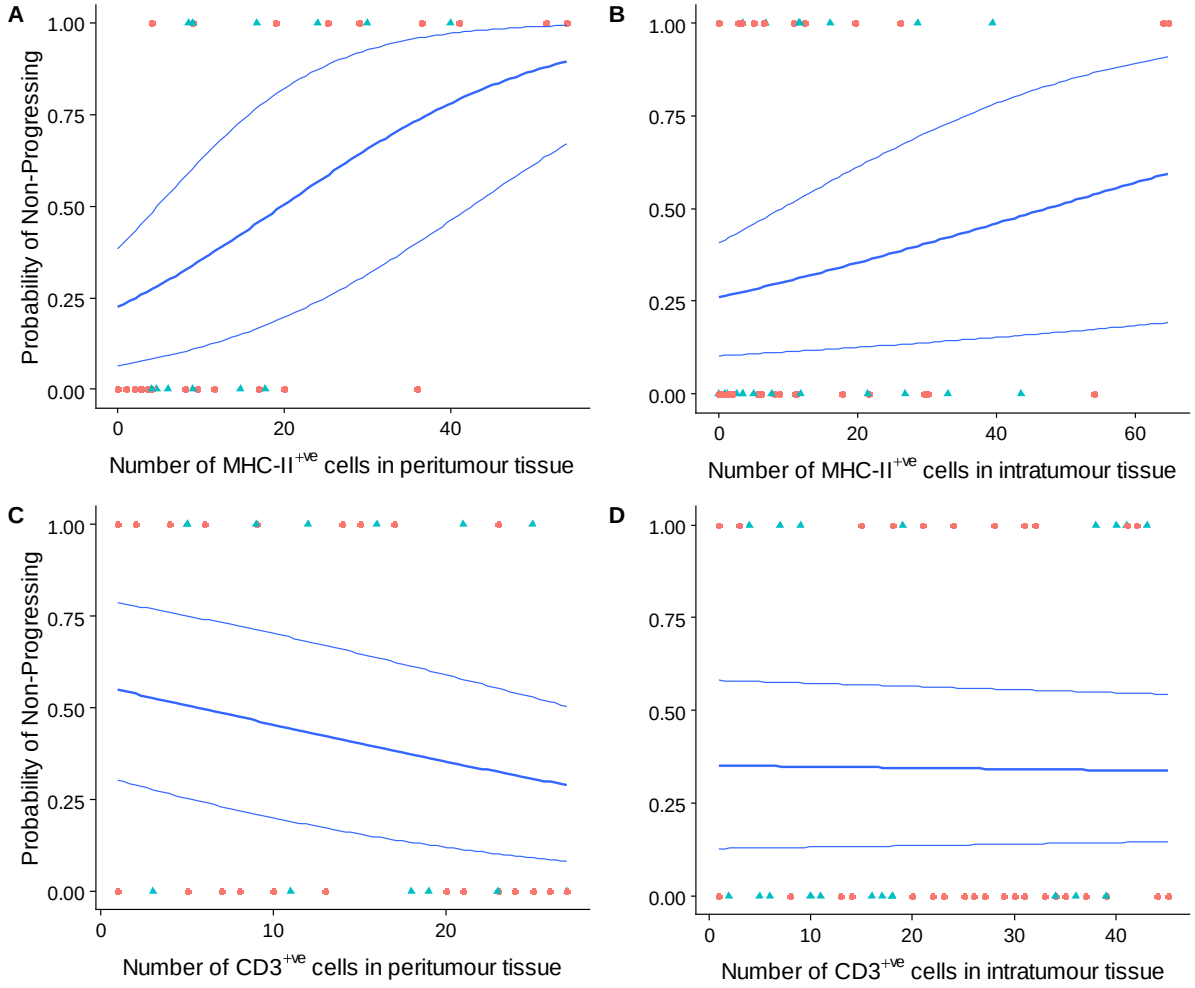

Figure 5-2 Probability for tumours to be classified as "Progressing" and "Non-Progressing" by the histopathological findings in tumour sections: A) MHC-II $^{(+ve)}$ cells in peritumour tissue; B) MHC-II $^{(+ve)}$ cells inintratumour tissue; C) CD3$^{(+ve)}$ cells in peritumour tissue; and D) CD3$^{(+ve)}$ cells in intratumour tissue. Points in the graphs show the classification of the observed tumours: "Progressing" (0) and "Non-Progressing" (1) in male (light blue triangles) and female (orange circles) hosts.

5.4.Discussion

Overall our findings suggest that wild Tasmanian devils show signs of humoral immune responses against DFTD which may differ between males and females, while in infected individuals antigen presenting cells around tumour tissues may influence tumour growth rates. We found that the presence of specific antibodies (IgG) against DFTD showed a positive relationship with infection status in males while not in females. Once individuals were infected, the rate of tumour progression showed an inverse relationship with the number of antigen-presenting cells in peritumour tissues; their numbers associated with slower than expected growth rates in tumours. That DFTD progression is being influenced by the devil immune response suggests that, not only can a proportion of individual devils in the population recognise tumour cells and mount an immune response, but that devils may present some level of resistance to the cancer. These results, linking immune response to functional influence on tumour progression, expand our knowledge of the devil-DFTD system and have implications for the epidemiology and evolution of this cancer-host system.

DFTD is transmitted from infected hosts to naïve hosts by bites during social interactions. Social contacts involving biting increase with body size as individuals reach sexual maturity in both sexes (Hamede *et al.*, 2013). Our results reflect this pattern, with probability of infection increasing with head width. Sex differences in allometric growth curves explain why the exponential increase in infection probability occurs at a smaller body size in females and may also influence the body size and age at which males, but not females, are exposed to DFTD. Growth asymptotes at a smaller size and younger age in female (generally age 2 years) than it does in male devils (at least 3 years). Nearly all females will breed at sexual maturity in their second year, irrespective of body size, or in their first year in diseased populations if nutrition and growth rate are sufficient (Lachish *et al.*, 2009). Females, then, will be exposed to DFTD once they reach sexual maturity as they will come into oestrus, mate, and be exposed to injurious biting. Reproductive skew and male competition will dictate that larger males have greater access to females (Jones, unpublished data). Thus, larger males and, older males in disease-free populations, are more likely to gain mating, and thus become exposed to DFTD.

Production of DFTD-specific antibodies (IgG) is a consequence of activation of an immune response against DFTD (Pye *et al.*, 2018, 2016; Tovar *et al.*, 2017), and our results

suggest that this also differs between males and females. The statistical support for sex differences in the relationship between IgG and infection status suggests differences between male and female devils in immune recognition of tumours. In the case of males, there is a positive significant relationship between antibodies and the probability of presenting tumours which reaches 100%, at high values of MFI. In contrast, this relationship in females, although follows a positive trend, was not significant. Assuming that an increase in IgG levels relates to exposure to the tumour antigens (Kreiss *et al.*, 2015; Pye *et al.*, 2018). Our findings support that immune recognition of tumour cells differ between males and females. Potential explanations of this pattern may be associated to the level of antigenicity of tumour cells or timing of activation of the immune response in relation to the moment of clinical detection of tumours.

The significant relationship between the number of antigen-presenting cells in the peritumour tissue and tumour progression supports an interaction between tumour growth and the immune response of devils. Infection represent an important pressure on hosts to develop strategies to optimize their fitness (Ruiz-aravena *et al.*, 2018), therefore it is expected that resistance in the form of immune response against DFTD may emerge as one strategy that devils may mount to reduce the detrimental effects of DFTD. However, the emergence of resistance against the cancer may select for counter measures from the tumours to evade the immune response mounted by their host. In the first case, our findings may be interpreted as that an increased number of APCs in the peritumour tissue have a suppressive effect on tumour growth. However, an alternative hypothesis might be formulated from our findings supporting that fast growing tumour may reduce the recruitment of APCs in the surrounding tissues, and thus tumours could counter-act the resistance response from the host by modulating the response itself.

Evidence of potential resistance was recently described by Pye et al (2016), however the authors did not present direct evidence of the functional impact of the immune responses on cancer progression. Devils may mount an immune response by first recognising antigenic molecules in DFTD cells (Kreiss *et al.*, 2015; Tovar *et al.*, 2018) which may trigger antibody production, and eventually by stimulating a cell-mediated immune response against the growing tumour resulting in limitation of tumour growth and thus fitness (Kreiss *et al.*, 2015; Pye *et al.*, 2016; Tovar *et al.*, 2017). This chain of events in the activation of an immune response against DFTD infection is supported by experimental infections and immunotherapy

(Tovar *et al.*, 2017). Resistance is not widespread within the devil populations studied at present, however it is expected to be under selection pressure, and therefore increase its frequency in the future. In this context, individual heterogeneities in the capacity of devils to respond to the infection by DFTD may allow for natural selection to operate in favour of resistant phenotypes which may increase the spread of resistant genotypes in the populations over time (Margres *et al.*, 2018).

One of the most remarkable features of Devil Facial Tumour Disease is its capacity to escape the immune system of multiple hosts, and therefore prevent immune rejection (Siddle *et al.*, 2013; Siddle and Kaufman, 2013). This trait has been crucial for the evolution of transmissibility in this cancer (Siddle *et al.*, 2007), however immune escape is apparently not perfect (Pye *et al.*, 2016). Immune responses against DFTD have been induced in devils by immunisation of susceptible individuals with tumour antigens, demonstrating that the devil immune system is competent (Woods *et al.*, 2007) and that immune responses are usually prevented by the down-regulation of MHC-I molecules in tumour cells (Pye *et al.*, 2018; Tovar *et al.*, 2017). The ability of tumours to "hide" their antigens is a hallmark of cancer and has been proposed to be adaptive, and therefore a loss or decrease in its capacity will negatively impact tumour fitness (Cavallo *et al.*, 2011; Hanahan and Weinberg, 2011). This condition raises questions and provides room for further inferences about the benefits of MHC-I expression for cancer, which may differ according to whether tumours act as a community of cells in coordination or as a group of cells in isolation (Romero *et al.*, 2018). In the case of DFTD, if the tumour tissues act as coordinated cells, the expression of MHC-I, which will trigger an immune response from the host, may be adaptive or maladaptive in different scenarios. MHC-I expression will be adaptive when stimulation of immune response increases host tolerance by reducing tumour growth rates and host mortality while increases infectious time. As a result, the probability of transmission to a new host will be higher fixating the alleles behind (Roy and Kirchner, 2000). In contrast, the expression of MHC-I on tumour cells and the consequent induction of immune response may be maladaptive when it results in tumour remission and therefore in a reduction of tumour fitness. This scenario will change if DFTD tissues act as a heterogeneous cell population in which each cell optimizes its own fitness within the tumour. In this case, the expression of MHC-I can derive from individual cells or lineages within tumours, for which the benefit may come from a reduction of competition by inducing an immune response from the host (Gatenby *et al.*, 2009). Thus, the immune response would act similarly to therapies in cancer in which treatment results in

selection that favours cell lines resistant to the treatment (Dagogo-Jack and Shaw, 2017; Shlush *et al.*, 2017). Considering the clonal nature of DFTD (Murchison *et al.*, 2010; Pearse and Swift, 2006), we could expect tumours to act as coordinated entities since cells are highly similar in their genetic material, however the emergence of different tumour lineages may be explained by the heterogeneous interests among cells within tumours (Murchison *et al.*, 2012; Pearse *et al.*, 2012; Ujvari *et al.*, 2013).

5.4.1. Future directions

DFTD represents a strong selective force acting on populations of Tasmanian devils. Disease progression impacts the energetic budget of individual devils (Chapter 2), and in males, the energetic demand reaches levels similar to the mating season which is the most energetically costly time of year for males (Ruiz-Aravena *et al.*, 2018). Accordingly, traits that enhance tolerance and resistance to DFTD are predicted to be selected for in infected populations (Margres *et al.*, 2018; Ruiz-Aravena *et al.*, 2018; Storfer *et al.*, 2018). This functional link between immune responses and disease progression supports the idea that resistance to cancer happens in natural conditions and is likely to positively impact the fitness of hosts. Further research exploring immune responses in devil populations in relation to disease arrival, and the underlying mechanisms is paramount to understand the potential for evolution of resistance to cancer in devil populations.

5.5.References

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Chapter 6

General Discussion

CHAPTER 6. GENERAL DISCUSSION

6.1. Research summary

This thesis integrates the fields of veterinary sciences, ecological physiology and eco-immunology to understand the physiological dimension of an evolutionarily young host-pathogen interaction between the Tasmanian devil and its transmissible cancer. By using a field-based methodology and a combination of observational and experimental approaches, I explored the applicability of the well-developed host-pathogen theory (HPT) to the recently emerged DFTD. The HPT predicts the occurrence of phenotypic traits in both host and pathogen which may act to favour tolerance and resistance to infection in the hosts. In this thesis, I assessed the overall physiological impact of DFTD progression in wild devils, identifying patterns of tolerance and resistance. As DFTD progresses, the metabolic demand in infected devils increases and their overall health decreases. The pattern of decline in health is different for male and female hosts, with females tolerating the infection better than males. Differences between the sexes were also found in the immune response against infection by DFTD and general immune condition of devils. This thesis provides the first knowledge of patterns of tolerance and resistance to DFTD in devils and of their relationship to cancer progression. The results of the study have important implications for current conservation actions for the species, as well as to inform theory and to predict potential ecological and evolutionary trajectories that the devil-cancer system may follow. In this discussion, I will develop some of the ideas, and potential predictions that can be drawn from the results of four years of research.

6.2. On the metabolic extended phenotype of the devil-DFTD system.

The extended phenotype, a concept coined by the evolutionary biologist Richard Dawkins (Dawkins, 1982), takes relevance in the case of host-pathogen interactions. The conceptual idea proposed by Dawkins refers to how phenotypic traits are not just the result of an organism's genotype and its interaction with the surrounding environment, but it is also

result of an organism's genotype interacting with other genotypes, for example a pathogen genotype. From this idea, phenotypic traits in an infected host will depend on the host genotype, but also on the genetic background of the pathogen (Casadevall and Pirofski, 2001; Qiu *et al.*, 2014; Råberg, 2014). Therefore, host responses to infections may result from genetic differences among hosts, and/or from differing pathogen strains (Gagneux, 2012). In applying this concept to the devil-DFTD system, it is expected that the ecological performance of an infected devil will be influenced both by its own genetic background and that of the growing tumour. Having in mind that HPT predicts that hosts and pathogens, at the individual level, constantly compete to optimize their fitness, the resulting phenotype from their interactions will define their potential for selection on traits that may optimize both host and pathogen fitness (Boots and Bowers, 1999; Cronin *et al.*, 2010; Griette *et al.*, 2015; Hayward *et al.*, 2014; King *et al.*, 2014; Svensson and Råberg, 2010). These individual-based interactions would therefore favour host and pathogen genotypes in which their resulting extended phenotype may drive the system toward coexistence.

The enhanced metabolic demand found in devils infected with DFTD is expected to shape both devil and tumour life history. Life-history theory proposes that multiple organic functions relate in a trade-off and the optimal combination of them will depend on the ecological circumstances in which organisms occur (Stearns, 1976). While acquisition of nutrients by organisms is linear, their allocation to multiple organic functions (fitness components) occur in parallel, and therefore the use of energy in one function represents a cost of opportunity to another one (Lailvaux and Husak, 2014; van Noordwijk and de Jong, 1986). As a result, natural selection is expected to act in favour of strategies that optimize fitness according to the limiting resources (Bêty *et al.*, 2003; Jones *et al.*, 2008; Knutie *et al.*, 2017; Ricklefs and Wikelski, 2002; Thornton, 2008). How energy budgets are assigned to different functions changes along the lifespan of organisms. For instance, during early life, reproduction is suppressed since growth is the main energetic demand (Figure 6-1A) (Folkvord *et al.*, 2014; Grabowska *et al.*, 2011). As a result, reproduction and positive fitness occur only when maintenance costs associated with somatic growth have ceased.

The increased metabolic rate in devils as tumour burden increases represents a constraint for both devil and tumours at the same time, and therefore is expected to select for both devil and tumour genotypes that optimize their joint energy use and fitness. The consequences of the energy constraints on hosts and tumours may select for varying

strategies, for instance, reduction in host maintenance costs which might allow them to cope better with the demand from the growing tumour (Figure 6-1C and 6-1F). This may be the case with infected females, which may adjust litter size to increase their level of tolerance, in relation to the capacity of males to tolerate the infection. As metabolic costs in organisms are one of the main forces driving the optimization of adult body size and lifespan (Speakman, 2005), a potential consequence of DFTD in long-term infected areas may be the reduction of devil body size resulting from selection on early sexual maturity and breeding. Increased food resources consequent to severe population decline leads to increased growth rates of juveniles and up to 50% of females that are able to reach a threshold body mass in May of their first year of independent life, just three months after weaning, to be able to come into oestrus and breed (Lachish *et al.*, 2007). Plasticity in growth rates has allowed devils to increase the rates of precocial breeding in infected devil populations in comparison to healthy ones (Jones *et al.*, 2008; Lachish *et al.*, 2009). This strategy of early reproduction represents an energetic trade-off with early death by DFTD infection (Jones *et al.*, 2008; Lachish *et al.*, 2009) but would come at a cost on somatic growth (Johnson *et al.*, 2012; Wells *et al.*, 2017). This pattern is expected to select for fast growing/early reproducer individuals at a cost on adult body size (Hou *et al.*, 2008) or immunity consistent with the "live fast, die young" paradigm (Johnson *et al.*, 2012; Promislow and Harvey, 1990). From the tumour perspective, constraint in energy supply would directly affect its growth rate. As a result, tumour variants capable of growing at rates in synchrony with energy acquisition by the host may be favoured by surviving longer, and therefore increasing their transmission in devil populations.

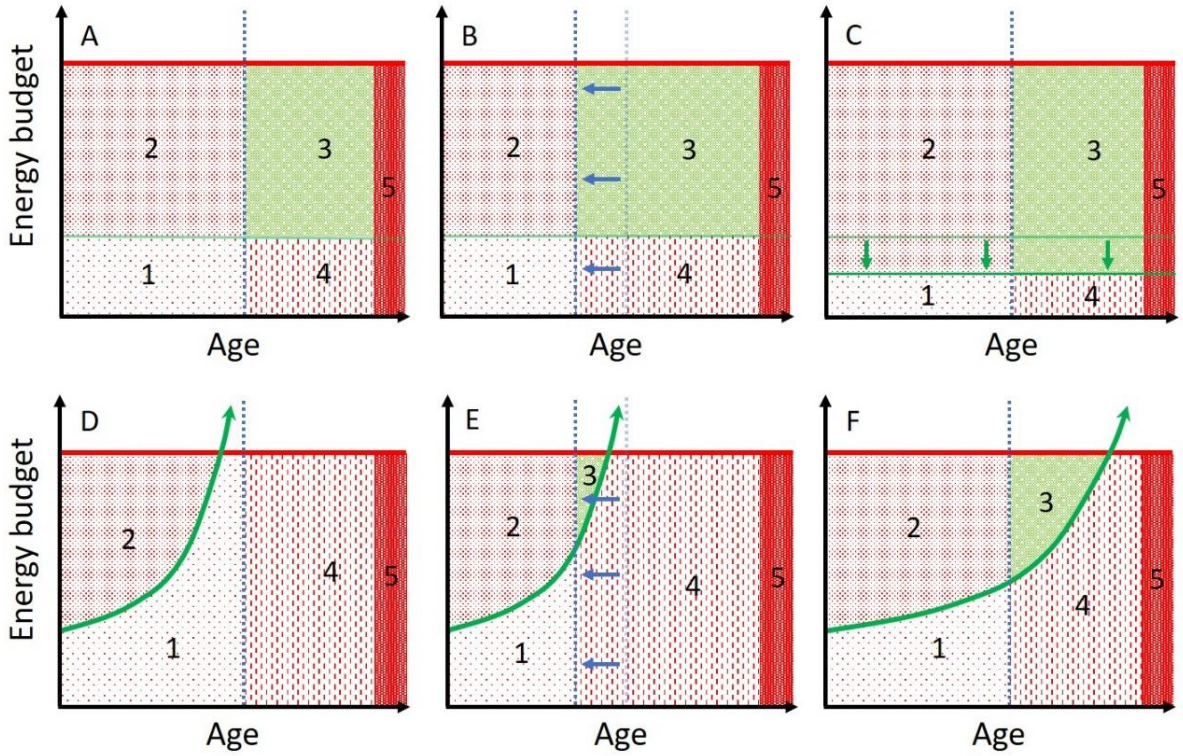

Figure 6-1 Scenarios of relationship between energy budget required/available for an organism and its age. Green lines parallel to the x-axis represent the minimum energy required to start reproduction, the upper red line shows the maximum energy budget available for an organism, and the blue dashed line parallel to the y-axis represents the age of first reproduction. The intersection of these lines represents different fitness scenarios (1 – 5) in the life of an organism. Area 1 represents scenarios of fitness 0, fitness may be positive in some contexts in areas 2 and 4, while area 3 represents the positive fitness in an organism's life. Area 1 = organism is too young, and energy available too low for reproduction; Area 2 = Energy available enough for reproduction, but organism is too young; Area 3 = energy available, and age enough for reproduction; Area 4 = organism at reproductive age, but low available energy; and Area 5 = Energy available, but reproduction is suppressed by senescence. A) Relationship of energy budget and age of an organism in "neutral conditions", with no selective pressure on energy allocation. B and C show maximization of fitness by plasticity in age of first reproduction, and energy required. Selection on energy allocation may operate on either or both strategies at the same time. Graphs D – F present scenarios of energy demand considering cancer progression, (green arrow). D) Energy demand increases as tumour burden increases at levels that do not allow for reproduction, driving host to death. E) Selection on plasticity of age at first reproduction allows host to present positive fitness. F) Selection on (s)lower energy demand from cancer progression allows host positive fitness. Selection may act on either or both strategies simultaneously.

6.3. Differential force and direction of selection is expected on devil and tumour traits.

The host-pathogen theory predicts contradictory trajectories in the evolution of the systems according to the traits in play. For instance, while tolerance in hosts may drive host and pathogen genotypes toward a stable evolutionary dynamic, the emergence of host resistance could trigger an "arms race" or "red queen" dynamic, which is highly instable (Best *et al.*, 2014; Roy and Kirchner, 2000). If there is spatial heterogeneity or sex differences in the emergence of traits, such as the ones described in this thesis for tolerance and resistance, are added to the system, the result is a mosaic of costs/benefit balance across traits that will vary across population (Gomulkiewicz *et al.*, 2000). How resistance and tolerance are theoretically expected to interact and how they might do so in the devil-DFTD system is explored in Figure 6-2.

The four traits that define a host pathogen system can be allocated along two axes connecting the host and pathogen genotypes (Figure 6-2). Tolerance is considered as a host trait, which relates in an inverse shape with virulence that is its pathogen counterpart (Little *et al.*, 2010). In the case of host resistance to infection, an increase in it translates in a reduction in pathogen transmission. Relationships between the traits also occur within host and pathogen genotypes, commonly in a trade-off situation. In the case of hosts, an increase in resistance is expected to trade-off with tolerance since a completely resistant host would not become infected, and therefore it would not experience any ecological pressure to cope with the infection (Råberg *et al.*, 2007). Consequently, no reaction norm between host health and pathogen burden can be built since pathogen burden remains at zero level in a complete resistant host. For pathogens, a similar trade-off might occur between virulence and transmission. Since transmission is influenced by how long a host may remain infectious and releasing pathogens to the environment, highly virulent pathogens that decrease host health too quickly result in a reduction of infectious time by hosts dying fast (Martin *et al. in press*). This results in a reduced transmission potential in comparison to a less virulent pathogen. From these last relationships we can build the last interactions between traits. Since virulence relates in an inverse shape with tolerance, the latter will relate positively with transmission by increasing the time in which hosts remain infectious (Roy and Kirchner, 2000; Martin, et al. *in press*). On the other hand, resistance will also relate in a positive way with virulence, since

when hosts become more resistant, a positive selective pressure for pathogens with virulence factors that increase their chances of transmission will be favoured (Gates *et al.*, 2014; Mackinnon and Read, 2004). Although tolerance and resistance are traits predicted by the HPT to emerge in host populations as a selective pressure by infections, whether they occur or not in the devil-DFTD system was completely unknown until their description in this thesis. Both traits are present in the devil populations and therefore are expected to be adaptive for devils. It is interesting that both traits, resistance and tolerance, may present sex differences which are expected to drive selection for tumours and therefore the epidemiology of DFTD in different directions by female and male devils. Thus, females, being more tolerant to DFTD progression, are expected to favour stable evolutionary strategies in the system by favouring less virulent tumours. On the other hand, the sharp decline of health experienced by males, that can reach about 25% of their body condition, may act against tumours infecting them by reducing chances of transmission. However, this outcome may change if males increase social interactions when infected to increase their chances of mating in a terminal investment effort. This scenario of terminal investment in male devils may be supported by the findings of larger size of sexual glands in late-DFTD infected males in relation to healthy males (Keeley *et al.*, 2012). Another potential consequence of the higher tolerance to infection in females is that they may act as super-spreaders (Martin *et al., in press*), or keystone hosts allowing the tumour lineages to persist in the devil population until the next mating season. This condition seems highly unlikely for males as their rapid decline in body condition compromises their survival after they become infected.

Resistance against DFTD in devils may select for higher virulent tumours. Host resistance involves a cost on pathogen fitness (Råberg *et al.*, 2009), which in the case of DFTD translates into a reduction in growth rates or even complete tumour regressions, as presented in chapter 5. This restriction of tumour fitness by devil immune response is expected, according to the HPT, to act as a selective pressure on tumour cells to develop strategies to avoid or modulate the immune response from its host (Mackinnon and Read, 2004; Siddle *et al.*, 2013). For instance, in the case of the oldest known transmissible cancer, the canine transmissible venereal tumour, the tumour tissue takes advantage of intimate manipulation of the host immune response to optimize its fitness by modulating its own growth rate. Normally undetectable by the dog immune system, CTVT presents an infinite replication capacity which may threaten the survival of its host as tumours grow, and therefore its own transmission. Thus, CTVT modulates its own growth and size by

stimulating an immune response from the dog host, thereby decreasing the risk of death for both tumour and host. This process is reversed by posterior down-regulation of antigenic molecules on the surface of tumour cells when the immune response threatens with clearing the cancer completely.

6.4. Evolutionary and adaptive medicine for devil conservation

Design and application of conservation strategies when outbreaks of an infectious disease are the dominant threat to a species is a challenging task. By default, pathogen eradication is the ideal scenario when it comes to disease management. Examples of successful interventions to control disease outbreaks in livestock, and human health are vast, however in the case of wildlife they are scarce. One of the main challenges for controlling wildlife diseases is the applicability of conventional health management strategies in an imperfect way, since access to whole populations is logistically and economically prohibitive. A second challenge, this time from the biological perspective, emerges from the condition that any intervention to increase the fitness of the host species (usually the goal of conservation strategies) may incur costs for the pathogen fitness. This might result in an important biological constraint for long-term conservation, where the main goal is to assist species populations to become self-sustainable to persist in the wild. Genetic diversity is crucial for the adaptive capacity of populations and species. The classical view of the influence of genetic variability in the viability of populations have supported that the higher the variability the better. The low genetic variability that Tasmanian devils present in the MHC-I alleles has been the most supported hypothesis for the emergence and spread of DFTD (Pye *et al.*, 2015; Siddle *et al.*, 2013, 2007). Based also in the low genetic variability of devils, is the contention that the species was thought to present very limited capacity to respond to the selective pressure imposed by the disease (Morris *et al.*, 2013), however devil populations seem to be rapidly evolving, and potentially adapting to DFTD (Epstein *et al.*, 2016; Hubert *et al.*, 2018; Margres *et al.*, 2018). The rapidity of the evolutionary response in devils means that adaptation is operating on standing genetic variation, that is, the genetic variation already present in the devil's genome at the time of the DFTD outbreak (Epstein *et al.*, 2016). This means that, despite loss of genetic diversity during the Last Glacial Maximum (LGM) and the Holocene (Brüniche–Olsen *et al.*, 2018, 2014), devils have the capacity in their genomic diversity to adapt to this recent disease challenge. This body of

evidence inspires a new view of "The right genetic diversity is better than more diversity" in the case of the devil-DFTD system.

Theoretical and empirical studies support the notion that hosts, and pathogens interact in a very intricate way. Changes in phenotypic traits in the hosts act as ecological and evolutionary pressure for the expression of traits in pathogens, and *vice versa*. In this scenario, increases in host resistance may likely result in an arms race between devils and tumours due to the decrease in transmission which might favour the emergence of more virulent DFTD variants. Although some theoretical studies support that both resistance and tolerance to infection can occur simultaneously in host populations (Restif and Koella, 2004),

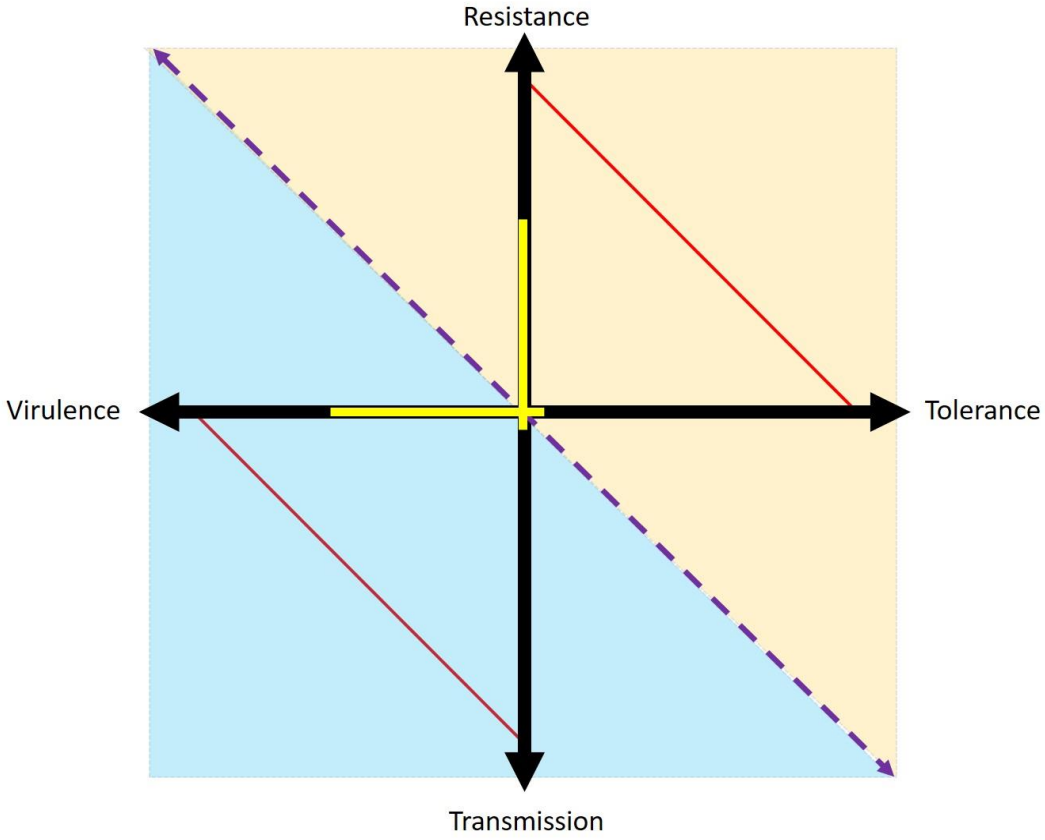

Figure 6- 4 Schematic representation of the interplay between the four traits identifiable in a host-pathogen interaction: Host resistance and tolerance to infection, and pathogen virulence and transmission. Traits allocated at the extreme of the same axis present inverse relationship (e.g. host resistance, translate in lower pathogen transmission). The large triangles represent whether traits depend on genotypes of host (beige) or pathogen (light blue). Red lines show the expected negative correlation between virulence and transmission in the pathogen genotype, and tolerance and resistance in the host genotype. The purple dashed lines show the positive correlation between host and pathogen traits. The extended phenotype of the interaction between host and pathogen genotypes can be visualized by the yellow bars along the axes. In the depicted example, the host – pathogen interaction presents high host resistance, low tolerance, low transmission rate, and high pathogen virulence.

the limited knowledge of these traits in the devil-DFTD system restrains the capacity to foresee the impact of actions to booster resistance or tolerance in devil populations. The current efforts to develop a vaccine against DFTD and the ongoing trials releasing immunised captive and semi-captive bred individuals into the wild are likely to change the transmission dynamic of DFTD by increasing resistance in hosts. How these management actions will affect the ongoing evolution process between devil and tumours remains to be elucidated, however a potential increase in tumour virulence is a likely outcome.

Regardless of the strong predictions of devil extinction due to DFTD outbreaks that early epidemiological models supported, Tasmanian devils have persisted in the long term in infected areas. Demographic compensation, driven by earlier sexual maturity and consequential reproduction of juveniles, is one of the main mechanisms by which devils might be persisting (Jones *et al.*, 2008; Lachish *et al.*, 2009). Another potential explanation is the metapopulation structure (Siska *et al.*, 2018) and the relationship between sexual maturity and transmission. Thus, disease spread may fragmentate metapopulations and by geographical configuration isolate subpopulations in which adults are eliminated by DFTD-induced mortality, recolonization by healthy juveniles from near areas may support and rebuild devil numbers. Although these mechanisms may take place and could be included in epidemiological models of the disease, the models would still lack the potential adaptive component described in this thesis. Host heterogeneities in tolerance and resistance are very likely to influence host competence, which translates into changes in transmission dynamics (Martin *et al.*, 2016; Vazquez-Prokopec *et al.*, 2016). Therefore, given that transmission is a key component of infection, considering how tolerance and resistance influence the epidemic behaviour of DFTD by influencing the physiological and behavioural dimensions of host competence is paramount to forecasting the future of the Tasmanian devil in the wild.

An increasing body of evidence, from both genomic and phenotypic studies, suggests that evolution can happen over short timescales, contrary to the classical view of natural selection acting on scales of millions of years (Kinnison and Hairston, 2007; Levis and Pfennig, 2016; Smith *et al.*, 2014). The devil—DFTD system demonstrates that these changes can occur as fast as in four to six generations (Epstein *et al.*, 2016; Margres *et al.*, 2018), which is much more rapid than, for example, the classic case of the European rabbit—Myxoma virus system in which rabbits evolved resistance over 20 generations (Di Giallonardo and Holmes, 2015). With evolution occurring in the DFTD-devil system, and the

evidence supporting that both tumour and devils are changing (Storfer *et al.*, 2018), the preservation of adaptive capacity of the system is a crucial new view and goal to be factored into strategies to preserve the species. This new framework based on the concept of "adaptive conservation medicine" provides a potential next-generation thinking for management of infectious diseases of conservation concern.

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